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EXPRESSION AND CHARACTERIZATION OF CHIMERIC MOUSE/HUMAN MONOCLONAL ANTIBODIES WITH SPECIFICITY FOR *Pseudomonas aeruginosa* PILI

By

JOUD SHAFIQ ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EXPRESSION AND CHARACTERIZATION OF CHIMERIC MOUSE/HUMAN MONOCLONAL ANTIBODIES WITH SPECIFICITY FOR *Pseudomonas aeruginosa* PILI in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY.

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'Read,

In the name of thy Lord.

The one who created,

created man

From 'something that clings'.

Read,

and thy Lord

the Most Generous,

Who taught by the pen,

taught man what he did not know"

chapter 96, The Holy Qur'an

To Kefah and Juman,

Words can never do you justice!

To my parents, family and friends

For your continued love and support

ABSTRACT

Pseudomonas aeruginosa is an important opportunistic human pathogen, whose clinical manifestations are apparent in burn victims and ICU patients. Murine monoclonal antibodies (MAbs) with specificity for *Pseudomonas aeruginosa* are currently being developed to be used in passive immunotherapy against *Pseudomonas aeruginosa* infections. However, a number of factors limit the use of rodent MAbs in human therapy, most importantly the human anti-mouse antibody (HAMA) response. The project described in this thesis attempted to overcome this problem by genetically engineering chimeric mouse/human antibodies, where in the murine constant region was replaced by that of human. This represents one of the earliest such attempts at antibody engineering undertaken in the *Pseudomonas* field.

Five different hybridoma cell lines (all of which produced MAbs reacting with *Pseudomonas aeruginosa* PAK pili) were obtained from Dr. R. Irvin. The expressed heavy and light chain variable region sequences (V_H and V_L) of four of the five antibodies were obtained either by the construction and screening of cDNA libraries, or by using the inverted polymerase chain reaction. Three different mammalian expression vectors, differing in; constant region isotype ($\gamma 3$ or $\gamma 1$), and the presence or absence of specific introns were constructed and used. The isolated murine V_H and V_L sequences were cloned into these plasmids and transiently expressed in COS cells. COS cell supernatants were tested for antibody secretion and assembly by the use of sandwich ELISA and western blot analysis, and for binding affinity to PAK pili by competitive ELISA. Novel antibodies (with affinities higher than that of the original mouse P antibody) were also generated by expressing different chimeric light chains (of B, D and E) in combination with the Pγ1 chimeric heavy chain. 'Swapping' of the D antibody V_H and V_L domains between the human $\gamma 1$ and K constant regions was also performed, with little improvement on antibody affinity.

Future 'humanization' of these monoclonal antibodies should lead to useful therapeutic or even prophylactic agents for use in patients suffering from severe burns.

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ABBREVIATIONS AND DEFINITIONS

Ab	Antibody
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonicacid)
ADP	Adenosine-diphosphate
Ag	Antigen
aKa	Apparent association constant
AMVSRT	Avian myeloblastosis virus super reverse transcriptase
A-RT-PCR	Anchored-reverse transcribed-polymerase chain reaction
ATCC	American Type Culture Collection.
B	PK3B monoclonal antibody
BECs	Buccal epithelial cells
BSA	Bovine serum albumin
C	Constant region
cDNA	Complementary deoxyribonucleic acid
Cδ	Delta constant region
CDR	Complementarity determining region
Cevag	24:1 chloroform: isoamyl alcohol
CF	Cystic fibrosis
Cf	Final concentration
Cγ1	Gamma 1 constant region
Cγ3	Gamma 3 constant region
C_H	Heavy chain constant region
C_K	Kappa constant region
C_L	Light chain constant region
COS cells	Monkey fibroblast cell line that constantly expresses SV40 T

C-RT-PCR	Conventional-reverse transcribed-polymerase chain reaction
C-terminal	Carboxy-terminal
D	CD4-TT monoclonal antibody
D_H	Diversity
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
E	EXO-S monoclonal antibody
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
FCS	Fetal calf serum
FR	Framework region
H	Heavy chain
h	Hour
HAMA	Human antimouse antibodies
HRP	Horse radish peroxidase
ICU	Intensive care unit
Ig	Immunoglobulin
I-RT-PCR	Inverted-reverse transcribed-polymerase chain reaction
J	Joining region
J_K	Kappa light chain J-genes
Kbp	Kilo base (1000 base pairs of DNA)
kD	Kilo dalton
Klenow	The large fragment generated by limited proteolytic digestion of *E. coli* DNA polymerase I

L	Light chain
L-V	Leader peptide-Variable
LMP-agarose	Low melting point-agarose
MAb	Monoclonal antibody
min	Minute
NAD	Nicotinamide adenine diphosphate
NEB	New England Biolabs
NC	Nitrocellulose
NI	Nosocomial infections
Nt-sp	Nutridoma media supplement (Boehringer)
N-terminal	Amino-terminal
OD_{600}	Optical density at 600 nm
Oligo	Oligonucleotide
P	18 B-13-41 (Pertussis) monoclonal antibody
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.4% (v/v) Tween 20
PCR	Polymerase chain reaction
penstrep	A mixture of penicillin and steptomycin (Gibco)
pfu	Plaque forming unit
Pfu	DNA polymerase from *Pyrococcus furiosus*
rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcribed-polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulphate
ss DNA	Single stranded DNA
sups	supernatant

SV40	Simian sarcoma virus
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
USB	United States Biochemical
V	Variable region
VDJ	Variable-diversity-joining regions of the heavy chain
Vf	Final volume
V_H	Heavy chain variable-region
V_L	Light chain variable-region
V-region	Variable region
V_T	Total volume

CHAPTER I

INTRODUCTION

A. *Pseudomonas aeruginosa*

1. *History and Epidemiology*

The term *Pseudomonadaceae* is used to designate a family of Gram-negative rod-shaped bacteria. Microorganisms belonging to this family are highly adaptive to their environment, have minimal growth requirements, are versatile in their carbon and energy sources, and can grow over a wide range of temperatures (4°C-43°C) (1). *Pseudomonas* is ubiquitous in nature, and the organism can sustain itself in distilled water (2), mop water (3), soil, plants (4), and a wide range of other habitats. Certain species within the family *Pseudomonadaceae* are pathogenic in either plant, animal, or human hosts (1). Of particular interest in the present work is the human pathogen *Pseudomonas aeruginosa* .

Pseudomonas aeruginosa was first isolated in 1882 by Gessard, who initially named the organism *Bacillus pyocyaneus* (5). *Pseudomonas aeruginosa* is an important opportunistic human pathogen. In addition to causing clinical manifestations in individuals suffering from cystic fibrosis, AIDS, or cancer, the organism continues to be a serious pathogen in burn victims and ICU (intensive care unit) patients. Nosocomial infections (NI) refers to hospital acquired infections. *Pseudomonas aeruginosa* has been described as an important nosocomial pathogen, and the organism has been isolated from a number of medical devices that use water, as well as from hospital sinks, toilets, humidifiers, etc. (6). In ICU patients, the risk of acquiring a nosocomial infection is positively correlated with length of stay and with catheterization (7,8).

2. *Virulence factors and their role in pathogenesis*

Pseudomonas aeruginosa has a wide arsenal of virulence factors, which in combination with the host immune status determines the outcome of the host-microbe interaction. Rodent models, such as the burned mouse model (9), have been developed to mimic human diseases caused by *Pseudomonas aeruginosa* . The role of the various virulence factors in pathogenesis have been inferred by observing the experimental outcome in these animal models using *Pseudomonas* strains mutant in particular virulence genes, and comparing it to the experimental outcome when wild-type strains are used. A number of virulence factors have been described, including the following:

a. Exotoxins

Exotoxin A is a member of the family of adenosine-diphosphate ribosylating toxins (ADPRTs). Exotoxin A transfers ADP-ribose from NAD^+ to elongation factor 2, thereby inhibiting protein synthesis in eucaryotic cells (10-12). Exotoxin A has 10,000 times the toxicity of *Pseudomonas* lipopolysaccharide (13), yet at present we can only speculate about its precise role in *Pseudomonas* pathogenesis.

Exoenzyme S is another adenosine-diphosphate ribosyltransferase. Exoenzyme S does not transfer the ADPR molecule to elongation factor 2 but rather to a number of GTP binding proteins (14). Unlike Exotoxin A , Exoenzyme S is not found in all *Pseudomonas aeruginosa* strains (15), and it is less toxic than Exotoxin A (16). The actual function of this protein in pathogenesis is unclear, however, it is suspected to play a role in adhesion (17) (see below).

b. Proteases

Elastase and Alkaline Phosphatase (AP) are two metalloproteases secreted by most clinical and environmental isolates of *Pseudomonas aeruginosa* (18,19), with Elastase being the most active and abundant of the two (20). Two possible mechanisms by which proteases contribute to *Pseudomonas* pathogenesis include: 1) degrading host connective tissue, thus enhancing the spread of the pathogen (21), and 2) cleaving host immune proteins, thus affecting the host defense system (22). Recently a new bacterial

protein called las A has been found to enhance the degradation of elastin when found in combination with elastase and AP (23).

c. Phospholipases

Phospholipase C (PLC-H) is one of two hemolysins that *Pseudomonas aeruginosa* produces, the production of this hemolysin is regulated by phosphate conditions (24). PLC-H degrades phospholipids found in eucaryotic cell membranes but not those found in procaryotic cell membranes (25). While creating PLC-H mutants, another phospholipase was discovered, PLC-N. This s*Econd* phospholipase does not possess hemolytic activity (26). The exact role that these two lipases play in the pathogenesis of *Pseudomonas* infections is not clear at present. However, it has been suggested that PLC-H might function to hydrolyze the outside layers of the eucaryotic cell membrane, whereas PLC-N could be hydrolyzing the inner layers (27).

d. Exopolysaccharide

In cystic fibrosis and chronic lung infections, the initial steps of attachment and colonization are accomplished by nonmucoid forms of *Pseudomonas*, such as those found in the environment. However, a mucoid form of *Pseudomonas* is subsequently isolated from the lungs of these infected individuals (28). The mucoid appearance is due to secretion by the organism of an exopolysaccharide called alginate (29). In the lungs of cystic fibrosis patients this exopolysaccharide coating appears to help the organism evade destruction by the host immune system (30,31) (see below).

e. Pili

Pseudomonas aeruginosa has polar, retractable, type 4 pili (32). Each pilus is made up of pilin protein monomers (33). Clinical isolates of *Pseudomonas aeruginosa* express one of 7 different possible pilin proteins (34). Each pilin protein is a chain of about 144 amino acids, with an intrachain disulfide bridge linking amino acids 129 and 142 (35). The protein can be cleaved by trypsin into four fragments: cT-I, cT-II, cT-III and cT-IV, with cT-III containing the most immunogenic part of the protein (36). The

N-terminal end of the protein is highly conserved, whereas the middle segment is the least conserved and forms the immunodominant part of the protein (36,37). The C-terminal end contains the intra-chain disulfide loop and is semi-conserved--it is located at the tip of the pilus and is responsible for binding to host cells (38-40).

The function of the pili in pathogenesis seems to be in the binding or attachment of the microorganism to host epithelial cells (41,42). In healthy individuals, nonmucoid *Pseudomonas* does not bind to epithelia. However, upon tissue damage by acid treatment or intubation (43,44), and also in the case of cystic fibrosis, it becomes possible for these microorganisms to attach.

Pseudomonas pilin is directly involved in binding to epithelial cells, and a number of experiments indicate that most if not all *Pseudomonas* adherence is mediated via the C-terminal disulfide linked domain of the pilin protein itself. According to Irvin *et al.*, synthetic peptides homologous to the C-terminal part of the pilin protein could compete with purified pili in binding to buccal epithelial cells (45). In a recent paper by Farinha and colleagues, the pilin gene in *Pseudomonas* strain PAO was exchanged for a mutant pilin gene that had 11 new amino acids replacing the usual 9 amino acids found at the C-terminal end of the wild-type pilin protein. In an *in vitro* binding assay the now mutant PAO strain (although pili biogenesis was not affected) exhibited a significant reduction in its capacity to bind epithelial cells (2-6 bacteria/assay cell compared to 50 bacteria/assay cell for the wild-type strain). This was reflected in an increase in the corresponding LD50 for the mutant strain (46).

In terms of receptors on eucaryotic host cells, there is no conclusive evidence as to the nature of the receptors for *Pseudomonas aeruginosa* which are present on epithelial cells. A number of the respiratory system pathogens appear to bind eucaryotic glycolipids (47), and *Pseudomonas* may also fall into this category. Some studies have shown that the receptor for *Pseudomonas* pili contains carbohydrates, and in one case this was defined to be sialic acid (34,48).

Pseudomonas pilin and Exoenzyme S have been found to share certain binding properties, both were found to bind Asialo-GM1 (17,34,47), and the monoclonal antibody PK99H (originally raised against PAK strain pili) was found to inhibit the binding of Exoenzyme S to buccal epithelial cells.

3. *Clinical manifestations of infection by Pseudomonas aeruginosa*

As stated above, *Pseudomonas aeruginosa* is an important nosocomial pathogen. Infections caused by this microorganism are relatively uncommon, are usually hospital-acquired, and typically occur in patients suffering from one or more predisposing conditions and in whom prior use of antibiotics has occurred (49). This section will elaborate specifically on infections that cause high morbidity in particular patient populations, such as burn victims and individuals suffering from cystic fibrosis. These are the patients where passive immuno-therapy using humanized monoclonal antibodies against bacterial pili may have the greatest potential benefit by preventing initial colonization.

To begin more generally, *Pseudomonas aeruginosa* has been reported to cause bacteremia (49), endocarditis (50), as well as ocular (51), otolaryngologic (52) and bone/joint (53) infections. Gastrointestinal (51) and skin wound (54) infections have also been observed. Nosocomial *Pseudomonas aeruginosa* infections of the central nervous system are uncommon (55), whereas a relatively large proportion (11.7%) of nosocomial-related urinary tract infections are caused by *Pseudomonas* .

a. Burn wound infections

Gram-positive microorganisms were the predominant isolates in early burn wound infections. However with advances in treatment and antibiotic use, sepsis caused by gram-positive organisms has been replaced with that caused by gram-negative microorganisms, including *Pseudomonas aeruginosa* (56). Unfortunately the mortality caused by *Pseudomonas* sepsis is much greater than that caused by the other gram-negative pathogens (57).

In terms of pathophysiology, in burn victims thermal shock leads to immune suppression. Hageman factor is activated, thereby increasing the protease load in the system, leading to an imbalance between proteases and protease inhibitors (58). This imbalance causes the degradation of host defense proteins, leaving the host even more susceptible to infection.

The infection process in burns can be described as follows (59): after thermal injury the broken skin is inoculated with a number of different microorganisms including *Pseudomonas aeruginosa* . Ordinarily the patient is placed on antibiotics, but *Pseudomonas* is resistant to many antibiotics, which gives it a competitive advantage over the other organisms and allows for colonization. The *Pseudomonas* organisms establish themselves in burned skin by adhering to eucaryotic cellular receptors via pili (60) and probably via Exoenzyme S. Exotoxin A and bacterial proteases then break down the host tissues, providing *Pseudomonas* with nutrients, thereby allowing the organism to multiply and spread (61,62). Due to the presence of functional flagella and a capacity for chemotaxis (63-65), the *Pseudomonads* begin to move following a concentration gradient of nutrients, until they reach and then enter blood vessels, resulting in sepsis (59). The hematogenous spread of the *Pseudomonads* and the continued production of proteases, which also activate Hageman factor and further overload the system with proteases (66,67), leads to additional destruction of defense proteins which makes the host more susceptible, and finally unable to resist the bacteremia. Continued production of Exotoxin-A leads to inhibition of protein synthesis in several organs (68), primarily the liver, which makes the host unable to replenish its defense proteins and its protease inhibitors. This vicious circle of increasing bacteremia, unchecked protease production, and host immunosuppression ultimately leads to death, usually through multi-organ failure(69).

In addition to the infections described above, respiratory infections with *Pseudomonas*, such as *Pseudomonas* pneumonia (70), can also occur, typically in

hospitalized patients with predisposing conditions such as cystic fibrosis and/or tracheostomy (71).

b. *Pseudomonas* infection in cystic fibrosis

Cystic fibrosis (CF) results from the inheritance of a faulty sodium and chloride transport system. This leads to viscous, dehydrated secretions from exocrine glands in a number of organs; the way each particular CF case manifests itself depends upon which organ is most affected (72). The incidence of CF in the caucasian population is relatively high (1 to 2 per 4,000 live births (73,74)), making it an extremely common inherited disease (75).

The lung is one of the major organs where CF manifests itself, and pulmonary complications are the primary cause of mortality in CF patients that survive beyond the neonatal period (76). The presence of viscous sputum in the lungs predisposes to infection by *Pseudomonas aeruginosa* , and isolation of the organism from the sputum of CF patients is correlated with a poor prognosis (75). Unfortunately, once established, eradication of this microorganism from the respiratory tract of CF patients is extremely difficult (75). Similar to burn wounds, as CF progresses the type of microorganism that colonizes the respiratory tract changes. Typically the first organism to infect the CF lung is *Staphylococcus aureus* (77), but with continued survival and increasing age, *Pseudomonas aeruginosa* becomes the predominant pathogen (78,79).

Both bacterial virulence factors and host factors play a role in establishing *Pseudomonas* lung infections in CF patients. A number of bacterial virulence factors are thought to govern attachment of *Pseudomonas* to eucaryotic cells, including: pili (41,42), exopolysaccharide (alginate) (80), and exoenzyme S (17). Depending on the strain and the environmental conditions, not all of these factors are expressed at the same time, and they may vary in their relative importance over the course of an infection. In terms of host factors, it has been shown that tracheal cells from CF patients exhibit increased binding to bacteria in comparison to tracheal cells from healthy non-CF

individuals (81). In CF patients, the question of whether *Pseudomonas* attaches to epithelial cells or to mucin is not completely resolved. Direct attachment of *Pseudomonas* to epithelial cells has not been documented in vivo, however, *Pseudomonas* has been isolated from the mucus of infected CF individuals (75).

In CF, *Pseudomonas aeruginosa* proteases come into play in a way that is similar to that seen in burn infections (72). *Pseudomonas* proteases degrade host protease inhibitors such as α1 protease inhibitor (82), thereby creating an imbalance between the proteases and their inhibitors. This imbalance results in the degradation of a number of host defense proteins, making the system more susceptible to further infection. Within the lung tissue, *Pseudomonas* may actually take advantage of the presence of certain host proteases. Human neutrophils are responsible for up to 80% of all protease activity found in the airway sputum (83). The combined protease activity (that of *Pseudomonas* and host system) results in the destruction of host defense proteins and more damage to respiratory cells. This damage in turn results in better adherence for *Pseudomonas.*

As mentioned above, during the early stages of CF *S. aureus* colonizes the airways (77) and induces inflammation. This infection usually responds to antibiotics, but the inflammatory process itself facilitates further colonization, initially by non-mucoid forms of *Pseudomonas*. Later on in the process the *Pseudomonas* switches from the nonmucoid to the mucoid form (30), thereafter taking advantage of the exopolysaccharide for binding (80) and protection against host defense system (31). As the disease progresses and with switching to the mucoid form, a large segment of the *Pseudomonas* pathogenic arsenal is down regulated (Elastase being an exception) (84). It would appear that *Pseudomonas* 'takes on a low profile' in order to maintain a chronic infection in the CF lung. Another important immunological event which occurs during the evolution of *Pseudomonas* lung infections in CF is a switch in the isotype of the anti-bacterial antibodies produced. Opsonizing IgGs switch to non-opsonizing classes such as IgG2 and IgG4 (85,86).

4. *Therapy of Pseudomonas aeruginosa infections*

Based upon our knowledge of the steps involved in the establishment of *Pseudomonas* infections, we can employ strategies that block progression of the infection at one or more steps. Often a combination of strategies, working simultaneously on a number of different fronts, is most effective (87). Some of the available strategies are outlined below:

a. Antibiotics

Antibiotics were the first treatment employed to combat *Pseudomonas* infections, but unfortunately they have not always been successful, especially in patients with serious underlying disease. *Pseudomonas* is naturally resistant to many of the commonly used antibiotics, such as first and second generation cephalosporins and ampicillin (88). Furthermore, resistance to formerly effective antibiotics can develop during the course of treatment, resulting in treatment failure (89). The ability of *Pseudomonas* to overcome the action of many antibiotics can be attributed to a number of factors, such as the low permeability of the outer membrane (90) and the production of enzymes which inactivate antibiotics (91).

b. Anti-Inflammatory Agents

In CF patients a great deal of the damage to the lungs is due to the host inflammatory response. The use of nonsteroidal anti-inflammatory compounds has been tested with some positive results (92).

c. Protease Inhibitors

The effect of neutrophil (PMN) elastases in creating an imbalance between the proteases and their inhibitors has prompted the use of anti-proteases in *Pseudomonas* infections. One study using an $\alpha 1$ protease inhibitor delivered through aerosols to CF patients gave promising results (93).

d. Immunotherapy

i. Vaccines- A number of vaccines against *Pseudomonas aeruginosa* have been developed and tested in clinical trials [reviewed by Cryz (94)]. The earliest vaccines used LPS as the protective antigen, whereas subsequent vaccines have used almost every known virulence factor expressed by *Pseudomonas* (94).

When using vaccines as a mean of active immunotherapy, the desired result can only be achieved by immunizing immunocompetent individuals such as CF patients, type I diabetics, and burn victims. Sufficient time is required for the body to build up an immune response before the infection overwhelms the host. However, in the case of immunocompromised individuals (e.g. severely traumatized ICU patients or patients with severe burns, AIDS, or those receiving cancer chemotherapy) there is literally 'no time to wait', and the patient must be given passive immunotherapy if they are to be protected.

ii. Passive Immunotherapy- In defending itself against *Pseudomonas aeruginosa* , the body uses primarily opsonising antibodies (95) and phagocytosis (95,96). Over the years much work has been done on developing and producing protective opsonizing immunoglobulins for use in *Pseudomonas* infected humans.

In one early study, plasma was collected from human volunteers and alcohol fractionated, and IgGs specific for *Pseudomonas aeruginosa* were isolated and used in passive immunotherapy (97,98). However, this preparation (called Immune Serum Globulin or ISG) was unsuitable for intravenous use (88). A more purified form of ISG, known as Immune Serum Globulin for Intravenous Infusion (IGIV) was later developed [for more details, see Collins (88)]. In addition to IGIV, a more specific antisera has also been developed, in this case by collecting plasma from animals or volunteers that had been previously immunized with one or more protective *Pseudomonas* antigens. Alternatively, plasma has been collected from human volunteers known to have high titre antibodies against various *Pseudomonas*

aeruginosa antigens. Both of these latter preparations are called hyperimmune globulins (88,99,100).

A variety of antisera preparations have been tested for clinical efficacy in passive immunotherapy of *Pseudomonas* infections [these trials are summarized by Collins (88)]. One clear disadvantage to using this type of therapy is the cost. For example, it would be extremely expensive at present to give prophylactic antisera to every patient who enters the ICU. On another front, since the conventional treatment for *Pseudomonas aeruginosa* infections is still antibiotics, in order to adopt passive immunotherapy more generally, it would have to be demonstrated to be more efficatious than antibiotic therapy alone. Furthermore, any new preparation of antiserum or antibodies would have to be shown to be both as efficatious and cheaper than any existing preparation before it would be adopted.

iii. Monoclonal Antibodies (MAb)- In 1975, Kohler and Milstein revolutionized antibody production with the development of 'monoclonal antibody technology' (101). By immortalizing mouse antibody-producing cells in culture, it became possible to produce large amounts of a single, specific, homogenous antibody against any defined antigen (101,102).

To generate monoclonal antibodies, mice are first immunized with the antigen of choice. The antigen is processed in the spleen, generating T helper lymphocytes and eventually mature B lymphocytes and plasma cells with specificities directed against the immunizing antigen. Spleens from the immunized mice are harvested, lymphocytes extracted, and fused to a mouse myeloma cell line, the cell fusion process being promoted by polyethylene glycol (PEG). The resulting fused cells are called hybridomas; they possess genetic material from both the splenocytes and the myeloma cells. Successful hybridomas are those which retain the immortality and growth characteristics of the myeloma cells, together with the plasma cell's capacity to produce and secrete antibodies. Hybridomas are selected for by growing the cells resulting

from the fusion process on a special selective media called HAT (stands for hypoxanthine, aminopterin, thymidine). Any unfused splenocytes die after a few days in culture, since they are primary cells and have a limited growth potential. The unfused myeloma cells also die in HAT media, but for a different reason. HAT media contains the drug aminopterin which blocks normal de-novo nucleic acid biosynthesis, so in order to survive cells must utilize the hypoxanthine (and thymidine) in the medium through the nucleotide *Sal* vage pathway. The myeloma cells are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT), which is absolutely required for the nucleotide salvage pathway. The splenic lymphocytes are not deficient in HPRT, and by contributing this gene to the myeloma cell at the time of fusion the resulting hybridoma is able to survive.

Hybridomas which grow up in HAT are then screened for the secretion of the desired antibody using any one of a number of methods such as radioimmunoassay, ELISA, etc. The selected hybridomas are then cloned and re-cloned in order to obtain a uniform population of cells, all of which secrete high levels of the single desired antibody. The final resulting hybridomas are grown in bulk culture and antibodies collected from the culture fluid by precipitation and/or by affinity chromatography. Alternately the hybridomas can be injected into the peritoneum of irradiated mice, where they grow rapidly, generating tumor ascites. The ascites fluid contains large quantities of the desired antibody, and it can be harvested by peritoneal tap both prior to and at sacrifice.

Monoclonal antibody technology is cheaper and more reproducible than production of human hyperimmune globulins, and if necessary a combination of different MAbs against different antigens can be used to obtain the best therapeutic result. In terms of passive immunotherapy for *Pseudomonas aeruginosa* infections, several different mouse and human MAbs have been produced against a variety of *Pseudomonas* virulence factors (103-109). The vast majority of these MAbs are of murine origin, and

because with repeated use rodent MAbs are immunogenic in humans (because of their Fc and framework differences, see below), a need arises to re-engineer these murine MAbs to make them very similar to human antibodies, while still retaining their antigen binding characteristics. This can be accomplished by genetically engineering murine/human chimeric or 'humanized' antibodies, which is the subject of this thesis.

B. Immunoglobulins

1. *Antibody structure*

a. The basic structural model

Our knowledge of the structure of the immunoglobulin molecule has advanced considerably since Pauling's first model, proposed in 1940 (110). Porter, Nisonoff, Edelman and Thorbecke performed key experiments leading to the formulation of the basic structural model for antibodies (111-115). In 1972, Edelman and Porter shared the Nobel prize for their accomplishments in elucidating this structure.

To begin at the simplest level, an antibody molecule is made up of two identical monomers, with each monomer being composed of a heavy chain and a light chain (figure 1). The heavy and light chains are held together by noncovalent bonds and by a single cystine bridge. The two monomers in turn are aligned to form a dimer, which is held together by noncovalent bonds and by cystine bridges in the hinge region (111-115). Digestion of an antibody molecule with papain causes cleavage above the hinge region, and generates two FAB fragments and one Fc fragment, whereas digestion with pepsin causes cleavage below the hinge region, yielding an $F(ab)'_2$ fragment (figure 1).

b. The fine structure of the immunoglobulin molecule

i. Variable and constant regions- Based on amino acid sequences, the heavy and light chains can each be divided into two regions. The region closest to the N-terminus varies in sequence from one antibody to another, and hence is called the variable region (V_H and V_L, for heavy and light chains respectively), with hyper-variability located in three sub regions called 'complementarity determining regions', or CDRs (116). The

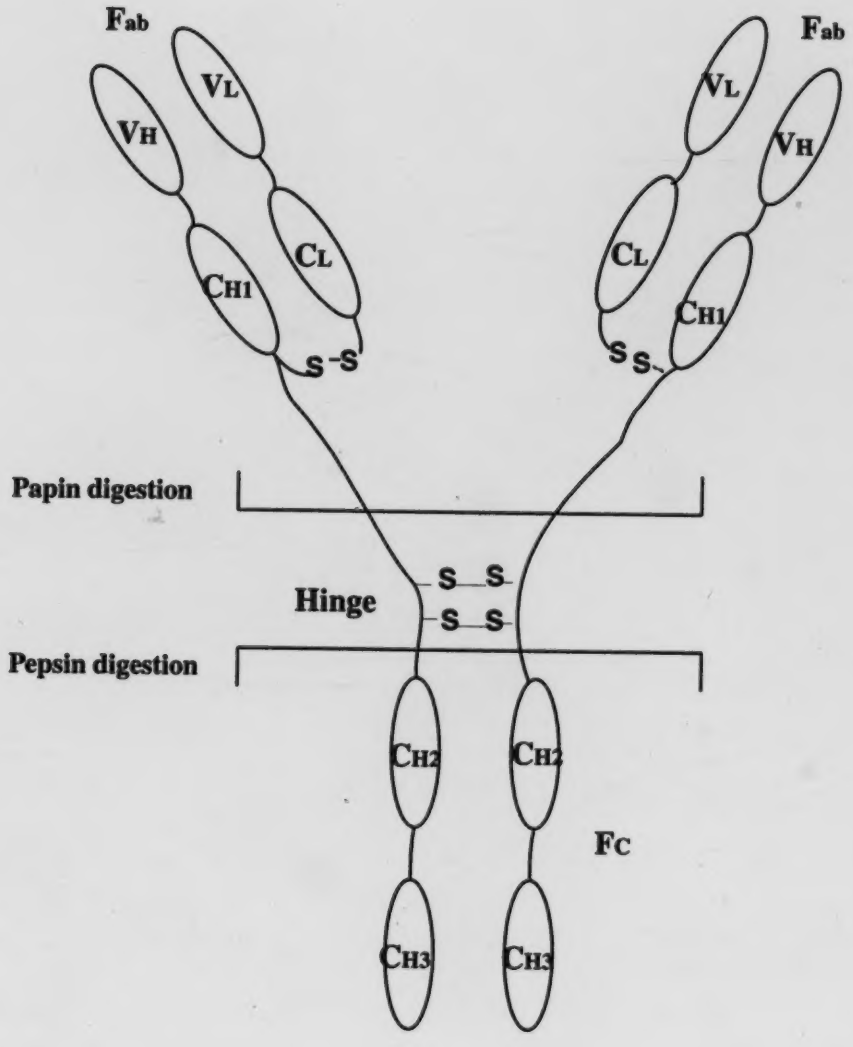


Figure 1. Schematic representation of a Human Ig G molecule, modified from Kabat (118). VL, VH are light and heavy chain variable regions. CL, CH1, CH2 and CH3 are constant domains of light and heavy chains. Location of the hinge region and sites of cleavage by papain (yields 2 Fabs and one Fcγ) and pepsin (yields F(ab)'2 plus multiple fragments) are demonstrated.

region located at the C-terminus is fairly conserved in sequence between immunoglobulins of the same isotype, and is thus called the constant region (C_H, C_L).

ii. The immunoglobulin domain- The immunoglobulin domain is the smallest building block in the immunoglobulin structure (117). Each antibody chain is made of a number of these domains, two in the light chain (V_L, C_L), and 4 or 5 in the heavy chain (V_H, C_{H1}, C_{H2}, C_{H3}, $\pm C_{H4}$), the number of C_H domains depending on the antibody isotype (117). Each of these domains is typically encoded in a single exon, and each is made up of about 110 amino acids, with an intra-domain disulfide bridge forming a loop of about 60 amino acids (figure 1) (118).

In terms of secondary and tertiary structures, each immunoglobulin domain is made up of two beta sheets (figure 2., seen best in the domain labeled C) which together form what is called an immunoglobulin fold or beta-barrel (117,119). The two sheets are stabilized by the single intra-domain disulfide bridge (117). Each sheet in turn is made up of three or four antiparallel beta strands, with connecting loops. The loops do not necessarily connect directly adjacent anti-parallel strands, for example, they can connect strands located on separate sheets (120,121).

The variable domain contains approximately 16 more amino acids than the constant domain, which results in two additional extended strands and a loop (117). Some of the loops which connect the V-region beta-strands were mapped to be hypervariable regions as described below.

c. The complementarity determining regions (CDRs)

The variable domains located at the N-terminus of both the heavy and light chains are responsible for binding to antigen (122,123). By simultaneously comparing a large number of the early immunoglobulin sequences, Kabat and Wu (116) were able to assign a 'coefficient of variability' for each amino acid position in the variable regions. By this analysis they came to recognize the existence of three hypervariable regions in each V-region chain, which they named the complementarity determining regions or

CDRs. The rest of the sequences, lying between each of the CDRs, were not as variable, and these regions were named the 'frame work regions' or FRs. As mentioned above, the CDRs can be assigned to some of the loops which connect the beta strands of the V domain, and the FRs correspond to the remainder of the residues which are present in the remainder loops and those responsible for generating the basic beta-sheet structure (117).

The three CDR loops contributed by the V_L domain are called L1, L2, and L3 (L1 being the closest to the N-terminus) and the three contributed by V_H are called H1, H2, and H3 (numbers same as for CDRs) (117). Although CDRs essentially correspond to hypervariable regions, their sequences are not completely random, since some constraints are placed on each loop structure, and therefore not all amino acids are possible at all positions (124). The conformation that each CDR loop adopts is largely related to the length and primary amino acid sequence of the loop (H3 being the most variable) (124).

2. *Generation of antibody diversity*

a. Organization of light chain genes

Figure 2 shows the organization of the light chain genes on the relevant chromosomes. The light chain protein is encoded by a number of genes; The L-V (leader peptide-variable) gene segments, one J gene (joining) and one C (constant region) gene contribute to the final transcript. Rearrangement of these genes is discussed below.

i. Murine light chain genes- Lambda light chains constitute only 5% of all the expressed murine light chains, and there is a correspondingly small number of Vλ genes. Vλ2 was the first immunoglobulin gene to be cloned (125); two additional Vλ genes were later found. Some Vλ genes are apparently rearranged and expressed more often than others (126,127). There are four Cλ genes, each with their own Jλ genes (figure 2), the J-Cλ4 element is apparently nonfunctional (126,127).

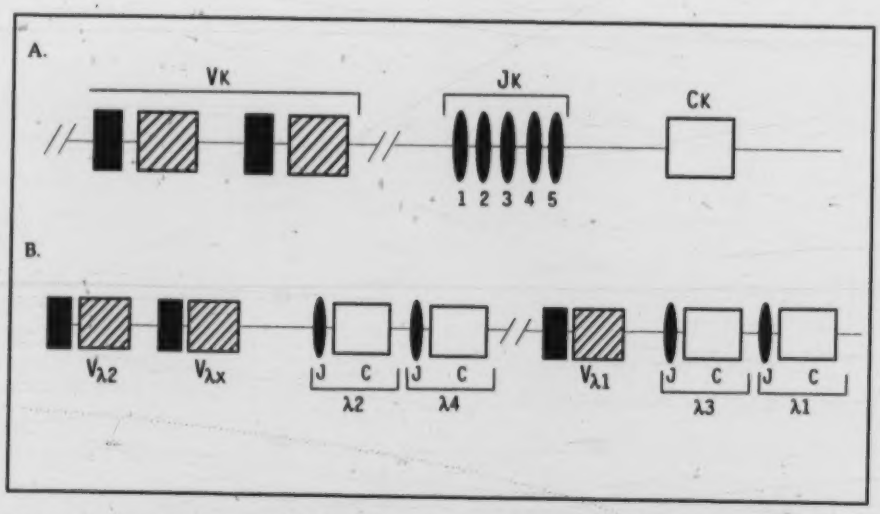


Figure 2. Organization of light chain genes. A. Kappa light chain genes, the same organization and number of Jκ and Cκ are present in humans and mice. Vκ represents variable region, every V exon ▨ with its own leader ▮ . Five Jκs are present, with Jκ3 being a pseudogene in mouse. B. Murine λ gene organization. Human λ system has a similar gene organization, except that all the Vλs are located 5' of the J-C region and there are more Vλ and Cλ genes than in mouse. Adapted from Max (138).

Kappa light chains constitute about 95% of all expressed murine light chains. Although the murine Vκ locus has not been studied as thoroughly as its human counterpart, there are 19 Vκ families (128), with each family containing many independent Vκ genes (128). Unlike the Jλ's, all five Jκs are clustered immediately 5' of the single Cκ gene; Jκ3 is apparently nonfunctional (129,130).

ii. Human light chain genes- In contrast to the mouse, 40% of all expressed human light chains are lambda. Of the estimated 70 human Vλ genes, only 24 are thought to be functional (131). Similar to the murine J-Cλ units, in the human genome every Cλ has its own J gene. A cluster of 7 J-Cλ genes has been mapped and sequenced, four of these are expressed, and the remaining 3 are pseudogenes (122,132). A second cluster of J-Cλ genes has been described, again containing both functional and non-functional genes (133). The human Vλ genes have been arranged into 7 families (118).

Kappa chains constitute 60% of the expressed human light chains. Of the estimated 80-85 Vκ genes, less than 50 are thought to be functional (134,135). These have been arranged into 4 families (118). As in the mouse, there is only one human Cκ gene, with a cluster of five Jκ genes immediately 5' (135,136).

b. Organization of heavy chain genes

Figure 3 shows a chromosomal map of the heavy chain genes. The heavy chain protein is a little more complicated than the light chain. In combination the L-V genes, DH (diversity) genes, and JH genes encode the leader peptide and the heavy chain variable domain (137). The constant region is encoded by one of a number of CH genes, depending on the expressed antibody isotype. Rearrangement of these genes is discussed below.

i. Murine heavy chain genes- There are over 10 VH families, containing an estimated total of 113 to 1086 different VH genes (138). To make up the variable domain, one of the VH genes, one of the ≈12DH genes (139,140) and one of the four JH

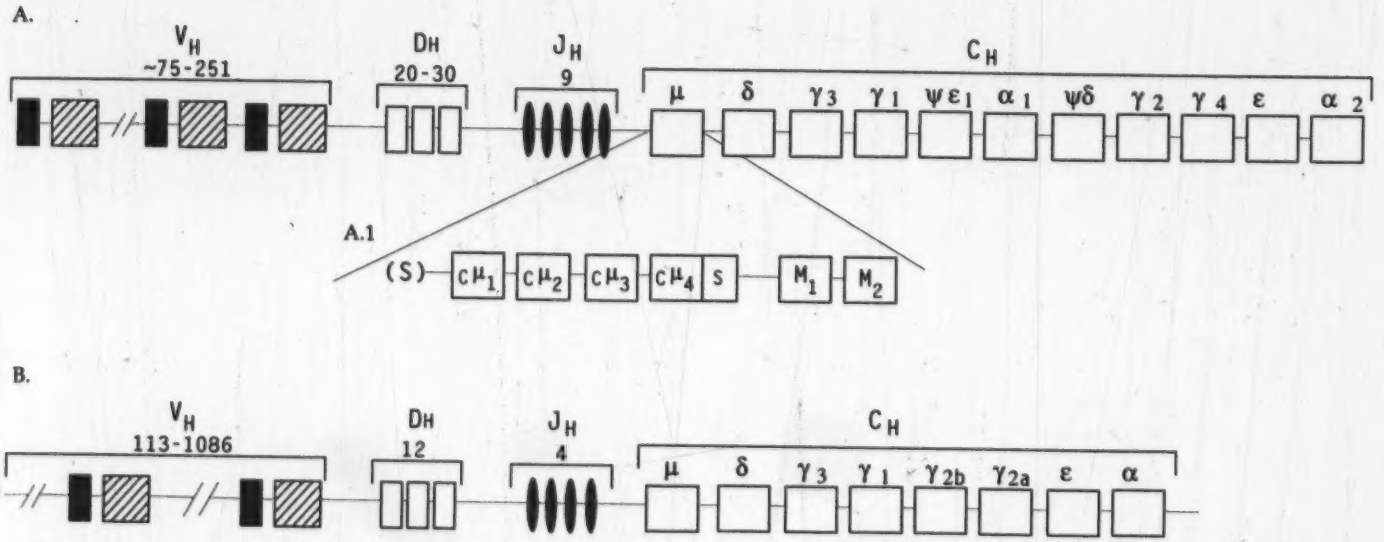


Figure 3. Heavy chain gene organization. A. human, B. mouse. Numbers above the boxes refer to the estimated number of gene elements in each case (see text). ψ indicates pseudogenes, with μ coding for the constant region of IgM, δ for IgD, γ for IgG, α for IgA and ε for IgE. Each one of the C_H boxes is actually made of a number of exons and introns as seen in A.1 for μ. M1 and M2 are exons coding for membrane bound forms of the antibodies. The s exon in Cμ4 is used when the secreted form of the antibody is produced. In IgG after the Cγ1 exon come exons for the hinge region, followed by the Cγ2 exon, etc. (S) switch sequence needed for isotype switching (present upstream of all C_H genes except for δ).

genes (141) are utilized. Depending on which antibody isotype is being expressed, one of the eight CH genes (142) will in turn be used.

ii. Human heavy chain genes- There are an estimated 75-251 different human VH genes, see Max for review (138). Over 30 DH regions have been found close to the JH region (143-145), which itself includes 9 JH genes, 6 of which are functional (143). Depending on which antibody isotype is being expressed, one of the corresponding 9 CH genes (there are 2 pseudogenes) will be used (146,147).

c. Variable-region gene rearrangements

Figure 4 illustrates the steps involved in light chain gene rearrangement and transcription/splicing. Variable region gene rearrangement occurs during B cell development [see reference (119,145) for review]. The variable region of the heavy chain rearranges first by joining one of the DH genes to one of the JH genes. This is followed by rearrangement of one of the VH genes to the fused DH-JH (148). The resulting VH-DH-JH is then transcribed and spliced to a constant region. If this results in a non-productive rearrangement (e.g. full length protein cannot be translated because of an 'out of frame' joining or an 'in frame' stop codon created at the joining), then rearrangement of the heavy chain on the other allele begins (148). However, if a full length heavy chain is produced, no rearrangement on the other allele will occur (e.g. allelic exclusion) (149,150).

Successful rearrangement of a heavy chain gene initiates light chain gene rearrangement, although these processes are not totally exclusive (151). Usually a VK will rearrange to one of the JK genes. If rearrangement fails on both kappa alleles, then the lambda light chain will rearrange (152,153).

By examining the sequences surrounding the different V-region gene segments, a unique DNA 'Recombination Signal Sequence' (RSS) was identified 3' of V genes, 5' and 3' of DH genes, and 5' of J genes (138). As shown in figure 5, a RSS is composed of conserved heptamer and nanomer sequences that are separated by a non-

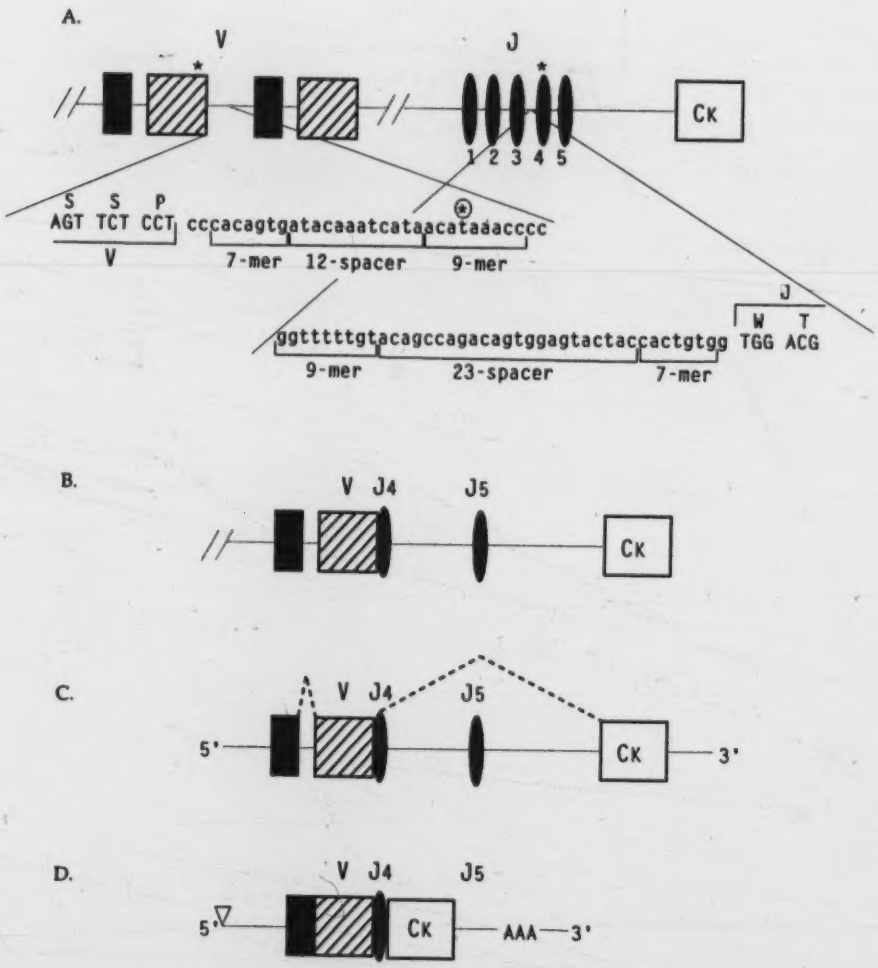


Figure 4. Kappa chain gene rearrangement. A. Germline DNA with recombination signal (RSS) sequences. [Adapted from Max (138)]. *The rearranging V and J. ⊛The t is usually an' A' in the consensus sequence of the 9 mer. B. Rearranged DNA (V-J joining). C. Primary RNA transcript. D. mRNA after splicing introns, 5' caping, and 3' poly A tail addition.

conserved segment of either 12 or 23 base pairs (129,130). In order for the proper VH-DH -JH recombination to occur, only gene segments with different spacer-sizes are joined (e.g. 12 to 23, but never 12 to 12). Suggested models for the recombination process, as well as the enzymatic machinery RAG-1 and RAG-2 have been reviewed elsewhere (145,151,154).

If every one of the VH genes has the same opportunity to recombine with any one of the DH genes, as well as with the JH genes, then an enormous amount of antibody diversity is possible, particularly since rearrangement happens in both heavy and light chains (although the light chain rearranges only the V and J elements). However there is another mechanism that further increases the level of diversity; that of flexibility in the joining areas.

d. Changes at the joining boundaries

In the light chain, there is flexibility in the joining of the VK-JK. In other words, recombination does not necessarily occur at specific defined nucleotides each time, but varies somewhat for each recombination event. A concrete example of this is seen in amino acid number +96 in the light chain. The VK starts at the +1 amino acid and ends at +95; JK starts at +97 and extends to +107. Therefore codon number +96 can be encoded by VK, by JK, or by a combination of both (118).

In addition to the flexibility in joining described above for the light chain V and J elements (and which also occurs at the VH-DH, and DH-JH junctions), the heavy chain has even more diversity generating mechanisms. Although the VH starts at +1 and ends at +94, it is difficult to determine precisely where the DH segment begins or ends (118). The DH genes themselves vary in length, as do the JH genes (118,138). In practice this means that depending on the particular rearrangement, a given DH segment can be 'read' in any one of 3 possible 'reading frames'. To put it another way, since the V-region of the heavy chain is made of three parts, if recombination at the VH-DH creates an 'out of frame' fusion (e.g. VH with respect to JH and CH), it can be 'corrected' by the DH-JH

fusion (155). It has also been reported that in some situations recombination occurred in such a way as to create a VH-DH-DH-JH fusion (138). Finally, many of the VH-DH-JH junctions contain extra nucleotides that are added to the joining area, either by the enzyme terminal deoxynucleotidyl transferase (uses no template, therefore called "N region" addition (156)), or resulting from the recombination process as template added nucleotides (called "P" nucleotides) (138). All of these nucleotide changes interestingly enough occur in the region encoding CDR3, and thereby have a direct effect on antigen binding.

e. Somatic mutation

All the above mentioned mechanisms which contribute to antibody diversity occur in the developing B cell prior to antigen stimulation. After gene rearrangement and repeated exposure to antigen, high affinity antibodies are produced through the process of affinity maturation (157), which is a result of somatic mutations and antigenic selection. Somatic mutations seem to cluster in the V(DH)J region in both heavy and light chains, with the mutation frequency going down the further 5' or 3' of the V(DH)J region one looks (158,159).

3. *Antigen binding sites and antigen-antibody interactions*

Of the 6 CDR loops which can potentially contact antigen (three H and three L), it appears that the heavy chain loops (and particularly CDRH3) are in closest contact with bound antigen. A naturally occurring, extreme example of this is the antibodies made by camels, which have no light chains at all, yet can still bind antigen effectively (160). In general, the number of CDR loops involved in antigen binding will differ for each different antibody. Wilson and Stanfield suggested that the minimum number of loops required is four (161), whereas Pessi *et al.*, were able to construct a "minibody" consisting of only three beta-strands and two loops; CDRH1 and CDRH2 (162). It is also important to remember that although CDRs have been implicated primarily in antigen binding (116,122,123), there are also examples of antibodies where one or more

FR residues are also crucial for antigen binding (163). See section below on CDR mimetics.

The idea has been put forward that the 'general topography' of the antigen combining site on an antibody can be correlated with the type of antigen the antibody is directed against (164). As summarized by MacCallum (164) and by Webster (165), antibodies against large antigens (e.g. proteins) have planar, relatively flat antigen combining sites, whereas antibodies against smaller antigens have combining sites which form more of a cavity (e.g. for haptens) or a groove (e.g. for peptides) (164,165).

Almost 90% of antigenic epitopes are discontinuous, which in the case of a protein antigen means that they are formed by a combination of side chains from amino acids which are distant from each other in the primary sequence, but which come together in three-dimensional space (166). A number of laboratories have reported that epitopes generally contain about 15 amino acid residues, but that interactions with only 5-6 of these are what provides virtually all of the binding energy (167,168). Regions of a protein with a convex shape, or with high mobility, or with a large negative electrical potential are more likely to elicit antibody responses (169,170).

Binding of antibody to antigen is a reversible process. The current idea is that this process follows an induced-fit model, rather than the older lock-and-key model (171,172). X-ray crystallographic data on Fv and Fab structures, crystallized both with and without bound ligand, indicate that upon binding conformational changes take place in both the antibody and the antigen, and that these changes generally appear to increase the strength of the interaction (171-173).

The strength of Ab/Ag binding is denoted numerically by the association constant (Ka), which is the reciprocal of the dissociation constant. In general terms a larger Ka signifies a stronger Ab/Ag interaction. In the simplest case, Ka designates strength of binding for a single antigen combining site associating with a single antigenic site.

However, because antibodies (and antigens) can be multivalent, the overall strength of attachment between the antibody and antigen (known as avidity) can be much greater than the affinity of a single Ab/Ag combining site. In terms of engineering recombinant antibodies, this means that avidity as well as affinity must be taken into consideration, since different constructs (e.g. Fvs versus intact antibodies; see below) will have different avidities. Clearly avidity is the more relevant measurement when considering therapeutic antibodies.

Cross reactivity is a common theme in Ag/Ab reactions; a single antibody may react with a number of different antigens (cross reactivity), but in general the affinity of binding will be different for each different antigen (174). Conversely, a number of different antibodies may bind to the same antigen, again with differing affinities (175).

4. *Antibody proteins- properties and effector functions*

a. Immunoglobulin proteins

i. An overview of the path from gene to secreted protein- As described above, prior to antigen stimulation V-region gene rearrangement occurs in developing B cells, resulting in the juxtaposition of V(DH)J elements. Transcription follows and the primary RNA transcript is capped, polyadenylated, and spliced to form mature mRNA which is transported out of the nucleus (figure 4, c and d). The mRNA is translated, and an amino-terminal leader peptide guides each antibody chain separately to the endoplasmic reticulum (ER), where the leader peptide is then cleaved from the main chain (119). In the ER folding and assembly of heavy and light chains occurs (119). A heavy chain ER retention protein (known as Bip) has been identified which binds to free heavy chains [this protein is displaced by the light chain as assembly proceeds (176,177)]. Polymerization (discussed below) also occurs in the ER, as does initial glycosylation (119). Following these events, the immunoglobulin molecule is transported to the Golgi system where glycosylation is completed, and the mature glycoprotein is finally secreted by exocytosis (119,178).

ii. Further details on the elements constituting immunoglobulin proteins-

Leader peptides- As mentioned above, both heavy and light chains are expressed with leader peptides at their amino-terminal end. These leader peptides or signal sequences range in size from 17-29 amino acids [by convention residues are numbered from -29 to -1 (118)].

V and C domains- The light chain consists of one variable and one constant domain, giving a total molecular weight of 25 kD (119). The heavy chain consists of one variable and 3-4 constant region domains, with a total molecular weight of 51-72 kD (119). As outlined above, the variable regions are composed of frame-work regions (FRs) and complementarity determining regions (CDRs). Assignment of amino acid positions to the various sub-regions of V_H and V_L are shown in table 1 (119).

C_H1/C_L pairing- Amino acids at the C-terminal portion of the V domain and the N-terminal portion of the C (heavy or light) domains are referred to as the elbow residues (117). This region of the antibody protein is flexible and allows for bending which facilitates binding to the antigenic epitope (117). The C_H1/C_L unit is thought to provide support for and facilitate the pairing of the variable domains (179). In addition to non-covalent and hydrophobic interactions, a disulfide bridge holds these two domains together, with the carboxyl-terminal amino acid of C_L (cys) participating in this bridge (figure 1) (119). The heavy chain ER retention protein Bip binds to the C_H1 domain (180); Bip can also bind to the light chain (181).

Hinge domains- Interestingly, the largest number of amino acid sequence differences between the various immunoglobulin isotypes are found in the hinge region (119). For IgA, G and D, the hinge region is an extended polypeptide that joins C_H1 to C_H2 (182). All of these hinge segments contain a number of prolines (which results in an extended conformation) as well as cysteines, which form interchain disulfide bonds holding the two heavy chains together (182). In IgM and IgE the 'hinge' is in

Table 1. Residues associated with the seven subsegments of the V_H and V_L domains [adapted from Kabat (118)].

Segment	Heavy chain	Light Chain
FR_1	1- 30*	1- 23*
CDR_1	31- 35*	24- 34*
FR_2	36- 49	35- 49
CDR_2	50- 65*	50- 56
FR_3	66- 94*	57- 88
CDR_3	95-102*	89- 97*
FR_4	103-113	98-107*

* Refers to possible insertions. C_L extends from residues 108-215, C_H from 114-496 (up to the end of C_H 3) or 114-628 (up to the end of C_H4).

fact an extra Ig domain (CH2) that contains hinge-like sequences. The hinge provides flexibility so that the antigen binding sites on the antibody molecule can adjust to the structure of dimeric or multimeric epitopes (183,184). The extended hinge regions in IgG3, IgA and IgD also make these antibodies more susceptible to proteolysis, which gives them a shorter half life *in vivo* and *in vitro* (table 2) (185).

CH2 domain pairing (CH3 for IgM and IgE)- This is an interesting domain since it possesses properties of both variable and constant domains (117). Unlike the CH1/CL domains, the two CH2 domains do not pair that well; no extensive contact between the two domains (117). Each CH2 domain is made up of two beta-sheets, with four beta strands per sheet, with changes in amino acids side chains that prevent dimer formation (with the other CH2 domain) (117). All of the beta strands in this domain are of intermediate length, between those found in VH and CH1/3 (117). All CH2 domains are associated with N-linked oligosaccharides, and the interaction between these sugars represents a major force holding the two CH2 domains together (117). This 'loose' pairing makes these domains more flexible and more accessible to the aqueous environment, which is consistent with the important role that the CH2 domains play in complement activation (see below) (179).

iii. Glycosylation of immunoglobulins- About 15% of all known variable regions are glycosylated (117). Glycosylation of asparagine residues within a CDR has been implicated in changing the conformation of the loop, thereby affecting the antigen binding properties of the antibody (186,187). O-linked glycosylation of serine residues in the hinge region helps to protect against proteolysis (188). N-linked glycosylation within the constant region (e.g. Asn 297 in IgG (189)) can affect protein conformation, as well as the capacity of the antibody to activate complement (190). The type of oligosaccharide added, and not simply the presence or absence of a carbohydrate moiety, affects the functional properties of the antibody (191). This is mirrored in the different carbohydrate composition of the different antibody isotypes (192).

Table 2. Effector functions and properties of Human Immunoglobulin Isotypes.

Property	IgG1	IgG2	IgG3	IgG4	IgE	IgA1	IgA2	IgM	IgD
H.C. molecular weight	51.000	51.000	56.000	51.000	72.000	58.000	60.000	72.000	64.000
Serum Concentration (mg/ml)	9	3	1	0.5	0.002	3	0.5	1.2	0.03
Approximate t1/2 (days)	21	21	7	21	3	6	6	5	3
Hinge region (length)	+ (23)	+ (19)	+ (70)	+ (20)	(C_H2)	+	+ (19)	(C_H2)	+ (36)
Effector function	-activates complement	-activates complement poorly	-activates complement	-binds $Fc\gamma RI$ and II	-primary response in allergy	-mucosal secrection		-primary response	-expressed on mature unstimulated B cells
	- binds $Fc\gamma RI$ II and III	- binds poorly to $Fc\gamma RI$ and II	-binds $Fc\gamma RI$, II and III		-parasitic infections -binds mast cells and basophils			-activates complement -powerful agglutinin	

* Adapted from Mayforth (119).

iv. Membrane bound immunoglobulins- All CH genes have two 3' exons (M1 and M2) which have the potential to encode a transmembrane anchor (e.g. see figure 3). To produce the membrane bound form of an antibody, the exon encoding the last CH domain is joined to the two membrane exons by differential RNA splicing (179). Together M1 and M2 code for a short hydrophilic segment (lies extracellular), a transmembrane hydrophobic segment, and a hydrophilic cytoplasmic tail (193). Although strong homology was detected for the transmembrane piece, otherwise the sequences of M1 and M2 differ for different antibody isotypes. This is especially so for the cytoplasmic segment, which is consistent with the idea that each different isotype of surface antibody transmits a somewhat different signal (194-196).

b. Characteristics and function of the various antibody isotypes-

i. The CH gene locus- Both the mouse (197) and human (147,198) CH genes have been mapped and sequenced (figure 4). Each constant region gene is made up of 3-4 exons coding for individual CH domains, plus a variable number of exons coding for the hinge region, the exact number depending of the antibody isotype. Downstream of each CH gene are the M1 and M2 exons discussed above.

ii. Isotype switching- VDJ joining occurs during the antigen independent stage of B cell development, and B cells thereafter express surface IgM and IgD. Following activation of B cells due to exposure to antigen, they switch from IgM and IgD expression, and instead express IgG, IgE or IgA. This process is known as isotype switching. The same unique VDJ element is transcribed, but it is spliced to a different CH gene (e.g. γ, ε, or α, figure 3) (179).

Several mechanisms have been proposed to explain isotype switching. Initially VDJ, Cμ and Cδ are transcribed as one long functional transcript, and through differential splicing, mRNAs for IgM and/or IgD are produced (199). To switch to the expression of another CH gene, a looping out and deletion model has been suggested, and experimentally 'looped out' (e.g. deleted) DNA circles have been isolated (200). So

called switch recombination sequences (S sequences, figure 4) are found 5' of every CH gene except IgD (201,202), these consist of arrays of tandemly repeated pentamers, which are thought to provide signals for the recombination process. Recombination between homologs or sister chromatids has also been suggested as a possible mechanism for isotype switching (reviewed in ref (203)). As for 'double producer' cells (e.g. cells simultaneously expressing more than one immunoglobulin isotype other than IgM and IgD), trans-splicing of RNA messages or cis-splicing of very long RNA transcripts, up to 100 Kb have been suggested (204). A description of the various signals and cytokines which regulate isotype switching is beyond the scope of this thesis, but these have been reviewed elsewhere (205,206).

iii. The various antibody isotypes- Table 2 summarizes some of the main structural and functional features of each of the various antibody isotypes found in humans.

IgM- IgM, initially in a membrane bound monomeric form, is the first immunoglobulin to be produced by an immature B cell. Upon antigenic stimulation a B cell gives rise to plasmacytes which secrete the pentameric or hexameric form of IgM. Due to multimerization, IgM often has a reasonably high avidity (total observed affinity). However, since IgM is a product of the primary immune response, the affinity of each individual combining site is usually low since the antibody has not undergone (or very little) affinity maturation.

Polymerization of IgM monomers to form a pentamer requires an additional glycoprotein called the J-chain. Amino acids at the C-terminal end of each μ chain extend beyond the CH4 domain, and these constitute the 'tail piece' which are involved with the J chain in multimer formation (117). In addition to providing a structural component, the J-chain plays an important role in regulating the formation of the IgM (and IgA) multimers (117).

IgG- In contrast to IgM, IgG is the dominant isotype made during a secondary immune response, and IgG antibodies generally have higher affinity binding sites because they have undergone affinity maturation. IgG antibodies are also the predominant isotype found in human serum, comprising 70-75% of the total immunoglobulin pool (207). There are 4 subclasses of IgG antibodies in humans, designated simply IgG1, IgG2, IgG3 and IgG4. According to Takahashi *et al.* (208), the four IgG subclasses have an overall

similarity of 95% at the nucleotide level, with the hinge segment being the least homologous part (e.g. there is only 60% similarity between the various nucleotide sequences in this region).

The hinge regions can be divided into three parts. According to Burton (209), the upper hinge extends from the end of CH1 up to (but excluding) the first cysteine that forms a disulfide bridge between the two heavy chains, the longer this region is, the more flexible is the molecule (210). The middle hinge, which varies in length between the different subclasses, contains all of the cystiene residues which participate in the formation of interchain disulfide bridges, along with a variable number of proline residues ["polyproline helix" (211)], this region can be thought of as a "relatively rigid rod-like double stranded structure" (182), and thus functions to stabilize the pairing of the two CH2 domains (212). The lower hinge connects the middle hinge to the CH2 domain, and is very similar in all IgG subclasses (182).

Other antibody isotypes- Some of the properties and functions of IgE, IgA, and IgD are also shown on table 2. Since they have little bearing on the subject of this thesis, the function and properties of these isotypes will not be considered further here.

iv. Complement activation- Complement denotes a group of serum proteins which can be activated in a cascade-like fashion to: 1) bind to target cells (e.g. bacteria) and significantly facilitate phagocytosis, and/or 2) initiate the formation of a membrane

attack complex, which results in the osmotic lysis of target cells (e.g. Gram-negative bacteria). Classical complement activation is initiated when the C1 complex (specifically C1q) binds to antigen-associated antibody. Residues Glu 318, Lys 320 and Lys 322 (all found within C_H2) are the sites of C1q binding to IgG (213). Interestingly, these particular residues are present on all four IgG subclasses, irrespective of their ability to activate (or 'fix') complement. However, experimentally only IgG3 and IgG1 are able to bind C1q, whereas there is no detectable C1q binding to IgG2 or IgG4 (214,215). Thus, in terms of complement activation, IgG3 and IgG1 are highly effective (214-217), most

probably because of the flexibility present in the hinge region of these molecules, as discussed above. The question of which IgG isotype (IgG1 or IgG3) is overall best at activating complement is less clear, and the results vary depending on antigen concentration, density and clustering of antigenic epitopes, as well as the nature of the antigen itself (182,214-217).

v. Antibody binding to Fcγ receptors and opsonization- The coating of bacterial cells with complement (C3 derivatives) and/or with antibodies greatly enhances the phagocytosis of bacteria in a process known as opsonization (193). However, this section will only focus on antibody-mediated opsonization. Opsonization occurs because Fcγ receptors present on the surface of macrophages and neutrophils allow these phagocytic cells to capture efficiently (e.g. via the Fc region of the antibody) the antibody coated bacteria (193). In humans, there are three different families of Fcγ receptors: FcγRI, FcγRII, and FcγRIII. FcγRI is the highest-affinity receptor for the Fc portion of IgG molecules, with FcγRII and FcγRIII showing somewhat lower affinity. In general Fcγ receptors bind with higher affinity to IgG1 and IgG3, but poorly if at all to IgG2 and IgG4 (193,218).

5. *Antibody engineering*

a. Motivation for engineering antibodies: limitations on the use of mouse monoclonal antibodies (MAb) in humans

i. Human anti-mouse antibody (HAMA) responses- Since the pioneering work of Kohler and Milstein in 1975, monoclonal antibody technology has revolutionized the fields of immunology and medical diagnostics (219-221). Unfortunately the use of mouse monoclonal antibodies as therapeutic agents has been much more limited, primarily because HAMA responses develop rapidly in patients receiving these antibodies. HAMA responses (220,222,223) accelerate the clearance of MAbs (e.g. shorten their half life (222)), and otherwise neutralize any potential therapeutic effects (220). In the face of HAMA responses, increasing doses of the MAb are required to achieve a therapeutic effect. Unfortunately this in turn increases the HAMA response, which finally limits any further use of the MAb.

ii. Limitations on effector functions- In addition to their immunogenicity, some rodents MAbs of certain isotypes are poor mediators of human effector functions (119,224).

iii. Limitations due to MAb size- The use of complete MAb molecules (e.g. secreted by hybridoma cell lines) as in vivo diagnostic or therapeutic agents is sometimes not necessary or even desirable. For example, intact antibody molecules may be too large to penetrate readily some areas of a tumor mass (225), whereas smaller molecules may be able to do so.

iv. Possible solutions- To overcome the problems described above for mouse MAbs, three general approaches have been adopted: 1) the production of human MAbs, 2) the production of recombinant mouse MAbs which have been specially engineered to be as 'human-like' as possible, and 3) the production of small antibody fragments. These approaches are described in more detail below (see also figure 5.).

b. Human monoclonal antibodies; approaches and problems

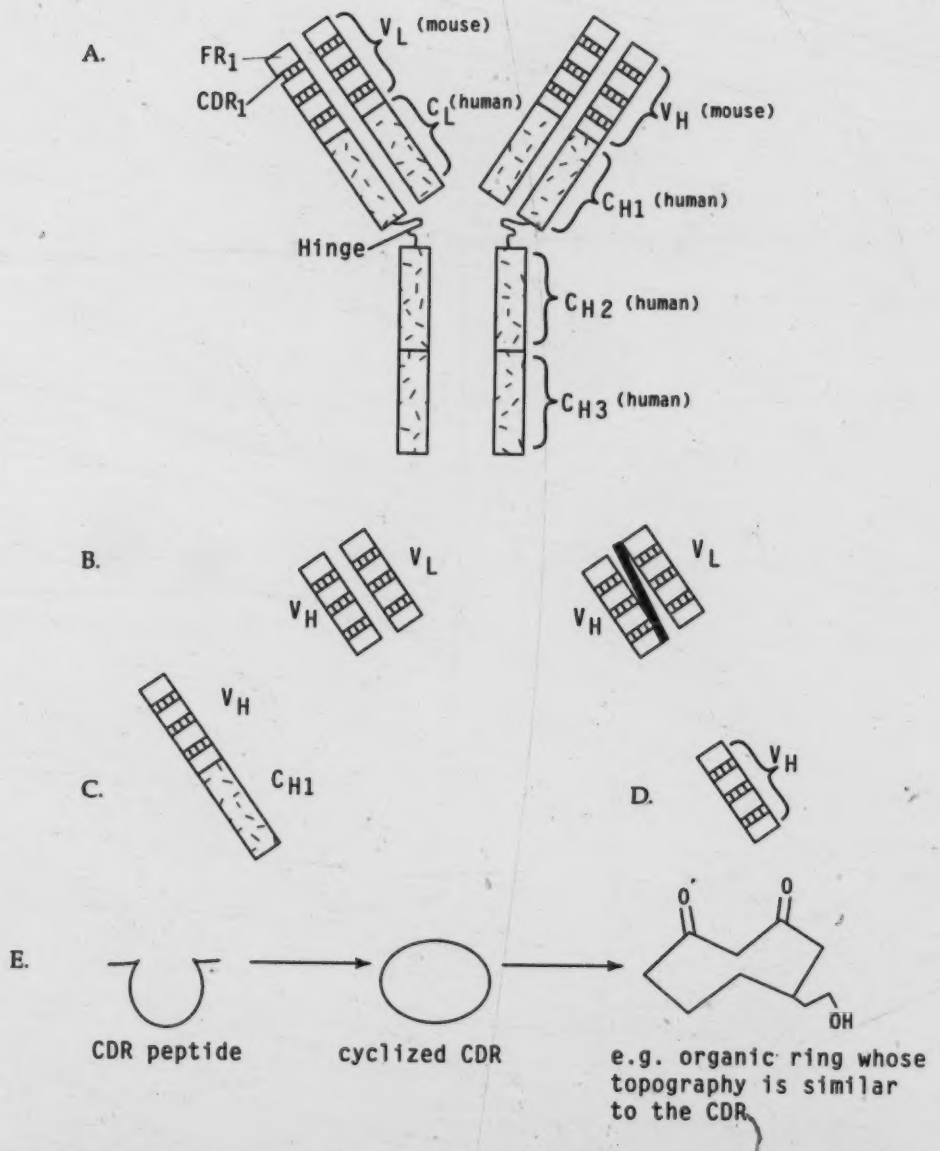


Figure 5. Recombinant immunoglobulin constructs. A. An intact immunoglobulin showing a mouse/human chimeric antibody. B. Fv (left) and single chain Fv (right). C. Fd, resulting from reduction and dissociation of a Fab D. dAb, consisting of only one heavy chain variable domain. E. Production of CDR mimetics. Peptides corresponding to the various CDRs are synthesized and tested empirically for binding to antigen. Those peptides which bind best are re-synthesized in such a way that they can be cyclized, so that their ends are constrained in a fashion similar to the ends of a CDR loop. The cyclized peptides are re-tested for binding, and if successful, their structures are solved and structural information may then be used to design an 'organic cyclized CDR mimetic', beginning with a generic ring structure that is carbon-based.

It is possible to obtain antigen-reactive B cells from human peripheral blood, fuse these with a mouse myeloma cell line, and generate mouse/human hybridomas by selection using the same strategy described above for mouse/mouse hybridomas (226). However, the generation of stable human hybridoma cell-lines secreting useful amounts of antibody is technically difficult, in large part because the human/mouse cells are unstable and tend to continually drop human chromosomes (119). In order to overcome some of these problems, researchers have more recently fused Epstin-Barr virus (EBV) transformed B cells to mouse myeloma cells--unfortunately, the resulting hybridomas almost always secrete IgM antibodies, which are more difficult to purify and lack many of the desirable effector functions of IgG antibodies (226,227).

In terms of the antigen specificities which can be obtained in human MAbs, it is of course unethical to immunize humans with most antigens, and therefore fusion of antigen-reactive B cells obtained from human peripheral blood can only generate specificities corresponding to those present in the individual donor at the time when they donated the blood (226). One potential approach to solve this problem has involved the use of 'in vitro immunization' (228). In order to increase the number of antigen-specific B cells prior to fusion with mouse myeloma cells, monocytes/macrophages, T cells, and B cells were removed from peripheral blood and cultured in vitro in the presence of antigen and cytokines. Although this technique is not particularly efficient, it has been used with success in a few cases. A second approach to generating antigen specific human MAbs has involved the use of humanized SCID (SCID/hu) mice (119,229). SCID mice lack endogenous T or B cells, but once their immune system is reconstituted with human lymphocytes they can be immunized at will with any desired antigen in order to amplify specific antigen-reactive human B cells. The desired B cells can then be harvested and fused to mouse myeloma cells as described above. A potential limitation of this latter approach is the fact that in SCID/hu mice a relatively limited

number of B cells with the desired specificity may be present in the reconstituted mice (230).

Another, related strategy to generate human MAbs has involved the creation of transgenic mice (231,232), in which the endogenous mouse immunoglobulin genes have been inactivated, and then replaced with large segments of DNA carrying unrearranged human immunoglobulin genes. Upon immunization, these mice produced human IgM and IgG antibodies specific for the immunizing antigen. The human immunoglobulin genes undergo typical V(DH)J recombination, show N-region nucleotide additions, and undergo affinity maturation.

Immunoglobulin phage display libraries are a recent technology which can potentially produce high affinity human MAbs. In this case RT-PCR is used to amplify the entire expressed VH and VL repertoires from human splenocytes or lymphocytes. The population of cDNAs is then cloned into a filamentous phage expression vector so as to fuse each VH and VL coding segment to the coat protein of the filamentous phage. Individual heavy and light variable genes are randomly combined, incorporated into the genome of an individual phage, and the VH and VL proteins end up being expressed on the surface of the phage whose genome contains the corresponding VH and VL genes. To select the desired VH and VL genes panning is performed. That is, the antigen of choice is fixed on plates, and the plates incubated with the entire 'library' of recombinant phage particles. Viruses which encode VH and VL proteins with higher affinities tend to stick to the antigen on the plates, whereas the remainder of the viruses do not, and these can be removed from the plate by washing. (The same effect can also be achieved by passing the recombinant phage particles over an antigen column.) Several cycles of panning can be performed to enrich for viruses encoding higher affinity V regions. Finally, the selected phages can be mutated randomly or in specific regions (e.g. mimicking affinity maturation), and the resulting 'sub-library' selected again by panning. Alternating cycles of mutation and selection can be continued until very high

affinity antibodies are finally generated. A number of variations to, improvements on, and applications of this technology have been reported (233-237). However, in practice some investigators have observed that the antibodies generated by phage display tend to be of moderate, rather than high affinity, and that repeated immunization of an intact mammalian immune system remains the most efficient method to generate high-affinity antibodies (B. Seed, personal communication).

c. Modifying mouse monoclonal antibodies to make them more human-like-

To tackle the problem of human anti-mouse antibody (HAMA) responses, researchers have used recombinant DNA technology to attempt to minimize the amount of mouse (e.g. foreign) material present in the mouse MAbs, replacing it instead with the corresponding regions of human antibodies. The key point is that all those parts of the original mouse MAb that are necessary for antigen binding must be retained in the final construct. Two general approaches have been taken; the generation of human/mouse chimeric antibodies, and the generation of 'humanized' antibodies.

i. Human /mouse chimeric antibodies- Chimeric antibodies consist of murine variable regions fused to human constant regions (figure 5) (238,239). Chimeric antibodies have several advantages: 1) they are much simpler and easier to create than are the humanized forms of antibodies described in the next section, 2) the affinity of the chimeric MAb will change very little from the original mouse MAb, since the complete intact murine variable regions are retained, and 3) chimeric MAbs are much less immunogenic in humans than are murine MAbs. The first clinical trial which reported using a chimeric antibody (240) involved 11 patients who were treated with a mouse/human immunoglobulin directed against a gastrointestinal tumor antigen. The plasma half life for the chimeric MAb was 6 times longer than that of the murine antibody, and only one patient developed an anti-MAb immune response. Even when a response does develop, it does not necessarily affect the therapeutic efficacy of the chimeric antibody (241). Since several murine heavy chain isotypes cannot mediate

effector functions in humans (242), an added advantage of chimeric antibodies is that they can be designed to contain human Fc regions with desired effector functions.

ii. Humanized mouse monoclonal antibodies- To further reduce HAMA responses, a second approach has been developed which essentially involves transplanting or 'grafting' the murine CDRs onto a human V-region framework plus C region. This is done separately for each heavy and light chain. In theory this results in an antibody which is entirely human except for the CDRs (243). However, because framework residues can also be important for antigen binding (244), Queen *et al.* (245) took the following approach to constructing humanized mouse MAbs: 1) they utilized the framework residues from a known human V_H or V_L whose framework sequences were most similar to the corresponding framework sequences of the murine chain under 're-construction', and 2) they used computer modeling to identify any framework residues from the parental murine MAb that either appeared important for maintaining CDR loop conformation, or came into direct contact with the antigen, these particular framework residues were also kept the same as in the original mouse antibody. Unlike chimeric antibodies, antibodies 'humanized' by this method usually end up having a lower affinity than the parental antibodies (246), and this, together with the sheer technical difficulties involved in design and construction, represent the major drawbacks of this approach . On the other hand, humanized antibodies are less immunogenic than either straight murine or murine/human chimeric antibodies. When the humanized antibody Campath-1 (directed against a human lymphocyte-surface antigen) was used in a clinical trial, no detectable antibody response was observed in 2 of 2 patients treated (247). Although the serum half life for humanized antibodies is longer than that for mouse (248) , 'anti-humanized antibody' responses have never-the-less been detected in some cases (249).

d. Small antibody fragments-

As mentioned above, intact antibody molecules may be too large to readily penetrate some areas of a tumor mass (225). In contrast, smaller recombinant antibody fragments (or fragments generated enzymatically from the original antibody) may be able to gain ready access to all areas of the tumor. As long as the smaller constructs still retain the original antigen binding capacity, they may well be capable of delivering the same therapeutic effect or diagnostic result as the whole MAb (250) and also be less immunogenic than the complete mouse MAb molecules.

Figure 5 depicts a variety of antibody-derived fragments which have been generated. A few of these have been produced historically by proteolytic digestion (251); however, production of antibody fragments using recombinant DNA technology is now the norm. This approach has made it possible to create a wide variety of constructs, and the availability of procaryotic and eucaryotic expression systems has made it possible to undertake large-scale production of some of these.

i. Fragments containing variable regions plus some portion of the constant regions- Fab and $F(ab)'_2$ fragments contain the V_H and V_L domains, together with the C_H1 and C_L domains (figure 1). In addition to being produced historically by enzymatic digestion, these were some of the first recombinant antibody fragments produced (252). A Fab fragment is about one-third the size of an intact antibody (~45 kD) (179), and it has monovalent binding. On the other hand a $F(ab)'_2$ is divalent for antigen binding, with the two combining sites linked by disulfide bridges (252). The advantages of these types of constructs include their smaller size and the relative ease with which they can be expressed in bacterial systems.

ii. Fragments that contain the H and L variable domains only- A further decrease in antibody fragment size was accomplished by producing Fv (253), scFv (254), and bi-scFv (255) constructs (figure 5). Fvs consist of V_H and V_L domains only. , however, since the two chains are held together only by weak interactions, in general these constructs are not stable, although there have been some exceptions (256). To

overcome the stability problem, a 15 residue [11-18 residues(119)] neutral peptide linker ($[Gly_4Ser]_3$) was designed to link covalently the V_H and the V_L (254), thereby creating the single chain Fv (scFv). The construction of scFvs is relatively straightforward compared to most other recombinant antibody constructs, in general the recombinant scFv proteins can be easily produced in bacterial expression systems, and these types of proteins have already been proven to be very useful as diagnostic reagents (250). However, the peptide linker might interfere ("obstruct") the antigen binding (119). In terms of in vivo diagnostic function (e.g. for tumor imaging) in general the smaller the antibody fragments are, the more rapidly they are cleared from the serum (e.g. scFvs are only 25 kD) (234,257). In practice it has been reported that the uptake of scFvs by a human tumor xenograft was comparable to the uptake of Fabs (257). In terms of potential therapeutic applications, the absence of a constant region in scFvs translates to an absence of effector functions; however, toxins and other proteins have been linked to scFvs to achieve a therapeutic effect (237). The avidity of binding of scFvs is reduced because scFvs are monovalent--this has been compensated for by constructing bi-scFvs (255), where a short amphipathic helix is added to the C-terminus of each scFv, which results in dimer formation (258).

iii. Fragments that contain a single variable domain- Attempts have been made to create even smaller constructs that still retain the antigen binding capability of the intact antibody. This approach is based on the idea that there is unequal participation of the CDRs in antigen binding, and that heavy chain CDRs are of primary importance in this process (161,259). For example Fds consist of only the V_H and C_H1 domains, and dAbs consists of only the V_H domain (figure 5, C. and D.) (260).

iv. CDR mimetics- (234,261) This approach takes the idea that there is unequal participation of the CDRs in antigen binding to the extreme, assuming that just one CDR (often CDRH3) is the major site of antigen binding. Peptides corresponding to the various CDRs are synthesized and tested empirically for binding to antigen. Those

peptides which bind best are re-synthesized in such a way that they can be cyclized, so that their ends are constrained in a fashion similar to the ends of a CDR loop (figure 5). The cyclized peptides are re-tested for binding, and if successful, their structures are solved using physical methods (e.g. NMR). This structural information may then be used to design an 'organic cyclized CDR mimetic', beginning with a generic ring structure that is carbon-based.

6. *Approaches to obtaining the cDNA fragments encoding VH and VL for mouse MAbs which are to be engineered*

Clearly the first step in engineering any recombinant MAb (chimeric or otherwise) is to obtain DNA/cDNA fragments which encode the V-regions of the parental mouse MAb. There are a number of ways to accomplish this task: 1) the construction and screening of genomic libraries, 2) the construction and screening of cDNA libraries or 3) the use of the reverse-transcribed polymerase chain reaction (RT-PCR).

The earliest experiments in antibody engineering depended on the construction and screening of genomic libraries (262-266). Working with large genomic DNA fragments is a lengthy and tedious process, however, some of the unique advantages to this procedure include: a) the absence of imposed nucleotide changes on the original isolated VH and VL sequences, b) the necessary signals needed for expression (e.g. promoters, enhancers, etc.) are often included, along with the VH and VL sequences, in the cloned genomic pieces (263) , and c) the occasional presence of naturally occurring restriction sites in the introns serve as cloning sites used for the exchange of the VH and VL segments.

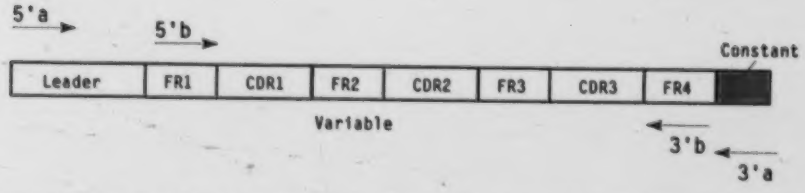
The construction and screening of cDNA libraries (267-269) offer a couple of advantageous over the genomic approach: a) due to the absence of introns, the cDNA clones are much shorter compared to their genomic counterparts; thus the probability of obtaining full length V-region sequences is higher, therefore a smaller number of cDNA clones have to be screened, and b) since the isolated cDNA of the VH and VL segments

originated from reverse transcription of RNA messages; the isolated clones usually represent those V-regions that are destined for translation and expression. However, some of the disadvantages to this technique are: a) due to the use of reverse transcriptase nucleotide changes can be imposed on the original V_H and V_L sequences (reverse transcriptase copying error) (see chapter 6), b) since the isolated V_H and V_L sequences were reverse transcribed from RNA messages, no promoter or enhancers will be included in the isolated cDNA fragments; such expression elements will have to be provided by the expression vector, and c) due to the absence of V-C introns from the isolated cDNA fragments, more technical maneuvers such as site directed mutagenesis (267,269) and connecting oligonucleotides (270) will have to be employed to fuse the isolated V_H and V_L to the appropriate constant regions.

With the invention of the polymerase chain reaction (PCR) , it became theoretically possible to amplify V-regions directly from the corresponding hybridoma cell line. RT-PCR also facilitates V-region cloning by the use of restriction-site containing primers. Templates used in V-region amplification were either cDNA reverse transcribed from total or polyadenylated RNA (271-274), or hybridoma cell genomic DNA (275,276). As shown in figure 6 when using cDNA as a template, cDNA synthesis can be primed with one of the following oligos : a) an oligo dT that will hybridize to the Poly A tail of the transcript (271-274), b) an oligo that will hybridize to sequences in the constant regions (277), or c) commercially available random hexamers (271). As for the oligos used in the amplification reaction; the sequence and location of the 5' and 3' primers depends on which of the following amplification reactions was employed: 1) Conventional -RT-PCR (C-RT-PCR), 2) Anchored-RT-PCR (A-RT-PCR), or 3) Inverted-RT-PCR (I-RT-PCR).

Shown in figure 6 is a schematic representation of C-RT-PCR. In order for this type of PCR to be possible, in general the target sequence to be amplified must be known so that two specific primers can be constructed, such as the 'b' primers.

A. Internal priming of first strand cDNA synthesis, showing possible PCR primers.



B. Oligo dT priming of first strand cDNA synthesis, showing possible PCR primers.

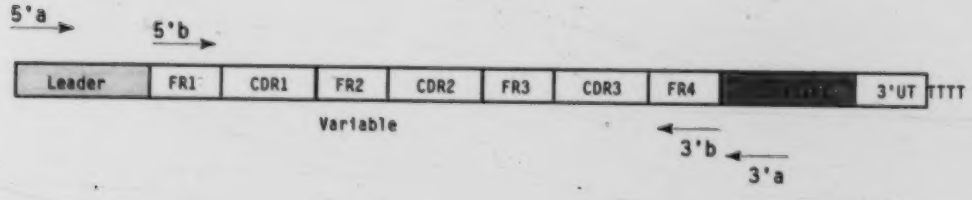


Figure 6. Schematic diagram of two possible strategies for first strand cDNA synthesis of V-region genes and possible locations of Conventional-RT- PCR primers. A. First strand cDNA when a constant region oligo is used to initiate synthesis. B. First strand cDNA when an oligo dT is used to initiate synthesis. The 5' and 3' b primers are those that hybridize to sequences inside the variable region. The 5' and 3' a primers are those that hybridize to sequences outside the variable region. FR refers to framework region, CDR to the complementarily determining regions, and 3'UT to the 3' untranslated region.

However, by definition the V-region sequence is not known at the time that the PCR experiment is initiated. Researchers have approached this problem by either: 1) designing primers hybridizing to 'consensus' residues located in the variable region (e.g. FRI and FR4) such as the 'b' primers , and/or 2) designing primers hybridizing to regions of known sequences (the constant region), or 'consensus' sequences (the leader) located outside at the boundary of the variable region, such as the 'a' primers.

The 'b' primers are made degenerate based on compiled sequences of variable genes (278), or as in the case of the 5' b primer, based on the amino acid sequence of the N-terminal part of the protein ((255), this work). Non degenerate universal primers have also been designed (279,280). The use of such 'inside' V-region primers makes it unnecessary to do a second amplification reaction in order to clone the V-region into expression vectors,assuming that restriction sites are built into the primers. The disadvantages to this approach are: 1) the possibility of imposing new amino acids on the original sequence (by FRI and FR4 primers) , and 2) the desired V-region sequences might be quite different than the FRI and FR4 primers and therefore amplification cannot occur.

If our primary target is to avoid imposing any amino acid changes on the variable sequence, then we must use the 'a' primers. Although the 3' a primers hybridize to the constant sequence making them easy to design, we do have some difficulties designing primers at the 5' end. To be sure to isolate any V-region by using a primer that hybridizes to the leader sequence, investigators designed degenerate oligos that (based on Kabat's compiled leader sequences) are supposed to amplify most of the known immunoglobulin leader sequences (274,277). However, the variable regions that we are pursuing could be missed and not isolated if the accompanying leader sequence was unique (which could happen fairly often). In addition, leader (and FRI) primers have been known to undergo abnormal priming inside the variable region (281). Using the C-RT-PCR for the isolation of V-region sequences, scientists have used the following

primer combinations: 'b' primers (255,278,279), 'a' primers (274,277,282), 5'b with the 3' a (272,283), and the 5' a with the 3' b (284). All combinations worked with various degrees of success.

Shown in figure 7 is a schematic representation of A-RT-PCR. A-RT-PCR introduces another solution to the problem of designing a 5' primer without imposing nucleotide changes on the V-region sequences. A tail of dGs is added at the 3' end of the cDNA molecule with the help of Terminal Deoxynucleotidyl Transferase. The dG tailed cDNA is then used as a template in an amplification reaction with the 3' primer hybridizing to the constant region and the 5' primer hybridizing to the dG tail (273,285,286). Another different , yet similar approach, was performed by Chen *et al.* (287). Instead of the poly dG tail, Chen *et al.* ligated a non-palindromic double stranded adapter on the end of double stranded cDNA molecules.

Another PCR method that offers a solution to the same problems addressed by A-RT-PCR, which also less technically demanding, is I-RT-PCR [(288), the present work]. As shown in figure 8 this type of PCR makes use of a more straightforward circularizing ligation reaction on double-stranded, blunted cDNA to place parts of the C-region both 'upstream' and 'downstream' of the V-region. This allows primers derived exclusively from the C region to drive the PCR amplification of the V-region cDNA. Other methods for the circularization of genomic DNA fragments have been developed by Zwickl *et al.* (289) and Jones (275). Zwickl *et al.* utilized naturally occurring restriction sites present around the gene of interest to circularize and then to linearize the DNA molecule for amplification (289), while Jones ligated adapters on genomic ends to close up the DNA molecule (275).

7. Approaches to expressing recombinant engineered antibodies

Once the necessary DNA segments encoding chimeric or humanized MAbs have been engineered and assembled, the recombinant protein must be expressed and tested to determine its functional properties. There are a wide variety of expression systems

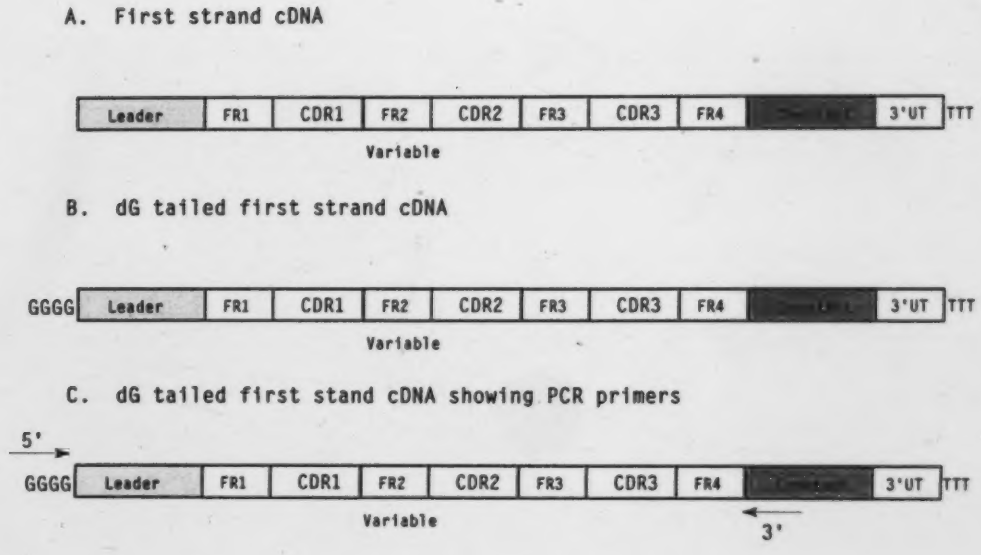


Figure 7. Steps leading to Anchored RT-PCR amplification of a V-region cDNA. A. Synthesis of first strand cDNA. B. Synthesis of a dG tail on the 3' end of the first strand cDNA molecule using Terminal Deoxynucleotidyl Transferase. C. Primers used in the amplification reaction. The 5' primer in this case consists of one or more restriction sites followed by $(dC)_{12-14}$ A constant region primer may also be used to initiate first strand cDNA synthesis as in Figure 6, and in this case the constant region primers used in the PCR may be nested (e.g. lie upstream of the primer used to initiate first strand cDNA synthesis).

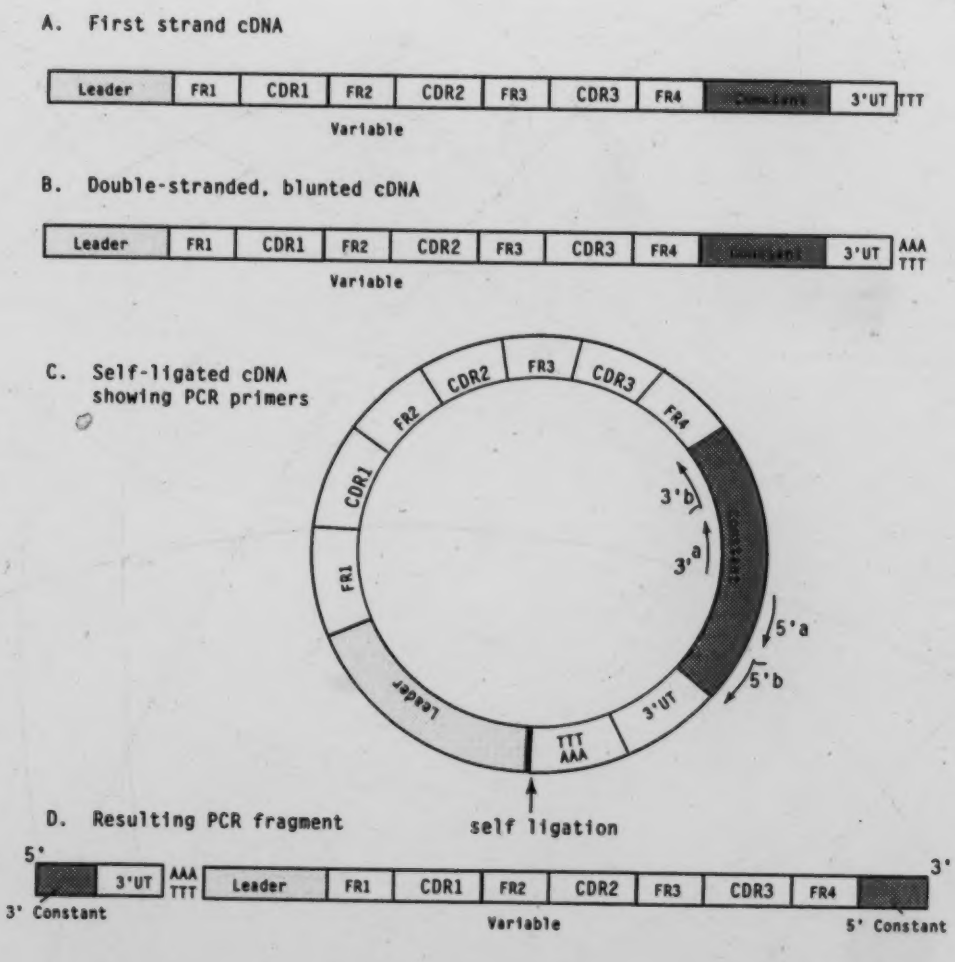


Figure 8. Illustration of the steps involved in Inverted RT-PCR amplification of a V-region cDNA. A. Synthesis of first strand cDNA. B. Synthesis of double stranded cDNA, followed by blunting of the ends of the cDNA molecule. C. Self ligation and circularization of the cDNA molecule. The amplification reaction is then carried out with primers a and/or b which hybridize near each end of the constant region and point 'outward' rather than 'inward' as do primers in Figures 6 and 7. D. The fragment resulting from the Inverted RT-PCR with the 3' end of the constant region lying upstream of the leader sequence.

available for this purpose (163,268,290-302).

Early immunoglobulin gene transfection experiments were done using lymphoid cells (122,263,290). The genomic DNA provided the necessary signals for expression (e.g. immunoglobulin promoters and enhancers), and these cells were known to be capable of assembly, glycosylation and secretion of the transfected antibody chains (263,290). Mammalian expression systems involve transfection of purified DNA into cell lines (such as CHO and COS) using one of a variety of methods (e.g. DEAE dextran complexed with DNA, electroporation, etc.) (268,303). If the cell line is highly transfectable and a large portion of the cells can be transfected, usable amounts of recombinant protein may be

generated over the first few days post-transfection, without applying any genetic selection to ensure that only the transfected cells survive in the culture. This approach is called transient transfection (used in the present work with COS-cells), and it can be contrasted with stable transfection [e.g. with CHO cells (294)], where for example relatively few cells in the culture are transfected, and those that are transfected must be selected over a period of weeks or months using a drug such as G418 or hygromycin, the resistance marker for which is incorporated into the transfected expression plasmid. Although stable transfection systems ensure that all cells in the culture are expressing the recombinant antibody, and ultimately they may give better yields of recombinant protein (in some cases by adding strategies to amplify the copy number of the transfected gene) the establishment of stably transfected mammalian cell lines is slow and labor intensive. In contrast, transient transfection into highly transfectable cells such as COS7 cells (163,267,268,295) is simple, rapid, and convenient. When these cells are transfected with plasmids containing an SV40 origin of replication, yields of recombinant protein (e.g. antibody) are typically sufficient to allow functional testing.

COS7 cells are an African green monkey kidney cell line, but modified by the incorporation of a portion of the SV40 genome into one of the chromosomes. In

practical terms what this means is that COS7 cells constitutively produce high levels of SV40 large T antigen, but there is no SV40 origin of replication present for the large T antigen to act on to drive replication. However, when COS cells are transfected with a plasmid containing an intact SV40 origin of replication, the large T antigen drives episomal replication of the transfected plasmid, resulting in the production of up to 1 x 10^5 copies of the plasmid within the nucleus of each transfected cell (303). When SV40 based expression plasmids are transiently transfected into COS cells, it is in part this 'episomal amplification' effect which results in a high copy number of the transfected gene, with consequent high levels of recombinant protein expression .

Some of the other eucaryotic expression systems used to produce recombinant immunoglobulin genes include: 1) insect cells via the baculovirus system (296), 2) Hela cells using the vaccinia virus system (297), and 3) plant cells (creation of transgenic plants) by *A. tumefaciens* and cross-pollination (298).

Microbial systems are inexpensive and can produce large amounts of some recombinant proteins, however they are not very effective in terms of glycosylation and assembly. In general microbial expression systems such as *E.coli* and yeast (300,301) are presently used primarily for the expression of smaller constructs rather than whole chimeric antibodies.

C. Statement of the rationale for and goals of the present research project

Passive immunotherapy of *Pseudomonas aeruginosa* infections using anti-*Pseudomonas* monoclonal antibodies has the potential to treat burn wound victims whose endogenous immune responses are significantly depressed. Several mouse monoclonal antibodies directed against *Pseudomonas* pili are currently available, and these may have therapeutic benefit both by blocking bacterial attachment and by promoting phagocytosis through opsonization, either by direct binding or by fixing complement. However, use of these murine monoclonal antibodies in humans will be limited by HAMA responses, and in addition, since most are of the IgM isotype, they

will not promote binding by human Fcγ receptors on phagocytic cells, nor will they be optimal for activating human complement *in vivo*. Re-engineering these murine MAbs to create chimeric mouse/human IgG3 or IgG1 antibodies should significantly increase their *in vivo* half-life and reduce HAMA responses, while at the same time optimizing *in vivo* complement activation and Fcγ receptor binding to human effector cells. Focusing primarily on the creation of chimeric antibodies rather than humanized antibodies should ensure that antibody affinities will not be significantly reduced, while at the same time significant practical benefit may be realized. The creation of chimeric antibodies is also less open ended than the creation of humanized antibodies, and therefore more suitable for a student research project. Even when creating chimeric antibodies, the function of the recombinant proteins must be carefully tested, and this can best be done initially by using material generated by transient transfection of COS7 cells.

The goals of the present research project were therefore as follows:

1) To obtain cDNAs for, determine the sequences of, and attempt to classify the V_H and V_L segments of five available murine MAbs which are directed against *Pseudomonas* pili.

2) To engineer chimeric mouse/human IgG3 and IgG1 MAbs from each of the five murine MAbs

3) To express the recombinant chimeric antibodies, prove that they are produced and assembled as expected, and determine their affinity for *Pseudomonas* pili.

4) To experiment with a novel approach in 'mixing and matching' the various available heavy and light chain chimeric antibodies, to determine if antibodies of higher affinity can be generated in some cases.

5) To experiment with a novel approach in 'swapping' V_H and V_L segments between heavy and light chain constant regions, to determine how such manipulations might affect antibody affinity.

CHAPTER II

MATERIALS AND METHODS

A. Mouse monoclonal antibodies and hybridoma cell lines

Mouse monoclonal antibodies (MAbs) and hybridoma cell lines were provided by Dr. R. Irvin, Department of Medical Microbiology and Immunology, University of Alberta. Hybridoma propagation, and monoclonal antibody purification was carried out as described by Sheth *et al.*(304). In all, five different hybridoma cell lines were provided, named simply PK99H, B, D, E and P. Properties of each hybridoma and the corresponding MAbs are described below.

The **PK99H** and **B** (original name PK3B) hybridomas were produced by Doig *et al.* (105) by fusing NS1 myeloma cells with splenocytes from mice that had been immunized with purified *Pseudomonas aeruginosa* 'K' strain pili (hereafter referred to as 'PAK' pili). The **B** MAb is an IgG which recognizes a subunit of pilin, it is strain specific in that it only recognizes K strain pili and pilin. The B MAb did not inhibit K strain *Pseudomonas* from binding to buccal endothelial cells (BECs), nor did it provide passive protection when given to mice which were subsequently challenged with a lethal dose of *P. aeruginosa* (304).

PK99H is also an IgG which is specific for K strain pili. Doig *et al.* (105) found that this MAb binds to the C-terminal portion of the pilin subunit, and the antibody and antigen binding site was further characterized by Wong *et al.* (305). In contrast to the B MAb, the PK99H MAb could inhibit PAK cells from binding to BECs (105), and in mouse models it was capable of providing passive protection against a lethal dose of *Pseudomonas* organisms (304).

The **D** hybridoma (original name CD4-TT) was generated by fusing NS1 myeloma cells with spleen cells from mice that had been immunized with the C-terminal portion of

Pseudomonas strain CD4 pilin conjugated to tetanus toxoid. ELISA assays showed that this MAb could recognize pili from a wide range of different *P. aeruginosa* strains. In mouse models, the D antibody also protected against a lethal dose of *P. aeruginosa* organisms (R. Irvin, personal communication).

The **E** hybridoma (original name Exo-s) was generated by fusing NS1 myeloma cells with spleen cells from mice that had been immunized with a synthetic peptide conjugated to tetanus toxoid. The synthetic peptide is homologous to a portion of the Exoenzyme S toxin of *Pseudomonas aeruginosa* , a part that displays 80% homology to the C-terminal portion of strain KB7 pilin. The E MAb was found to cross react with pili from a wide variety of strains of *P. aeruginosa* , and it could protect mice from a lethal dose of *P. aeruginosa* (R. Irvin, personal communication).

To generate the **P** (original names 18B-13-41, and Pertussis) hybridoma, Stenson immunized mice with whole killed *B. Pertussis* Bp347 organisms, and fused the resulting splenocytes with SP2/0 myeloma cells (306). By coincidence, this anti-*Pertussis* MAb was found to cross react with a wide range of *P. aeruginosa* pili, and to pilin synthetic peptides conjugated to BSA. It also protected against a lethal dose of *P. aeruginosa* in mouse models (304).

B. Isolation and analysis of RNA

1. Isolation of RNA and preparation of northern blots

RNA was isolated using the method of Chomczynski and Sacchi's (307). Agarose (1%) formaldehyde-denaturing gels (308) were used to analyze RNA samples, these were cast in a BioRad minigel apparatus, using a total gel volume of 50 ml. Gels were made by adding 0.5 g of agarose to 44 ml milliQ water, dissolving in a microwave, cooling to 65°C, and adding 5 ml of 10 X MOPS running buffer (0.2 M 3-[N-morpholino] propane sulfonic acid pH 7.0, 50 mM sodium acetate, 10 mM EDTA pH 8.0) plus 1.5 ml of 37% w/w formaldehyde (Fisher Scientific).

To prepare the RNA for electrophoresis, RNA samples (1-5 µl) were mixed with 15 µl of RNA treatment solution (1 X MOPS buffer, 6.5% (v/v) formaldehyde and 50% (v/v) formamide) +/- autoclaved water to take the total volume to 20 µl. The mixture was heated to 55°C for 15 min, cooled on ice, and then 2 µl of RNA loading solution (50% glycerol, 1 mM EDTA) and 1µl of ethidium bromide solution (10 mg/ml in water) were added prior to loading. To mark the progress of the electrophoresis, one empty lane was loaded with regular 10X DNA load dye containing bromophenol blue (308). Electrophoresis was carried out in 1 X MOPS running buffer, and continued at 70V for 1h or until the DNA load dye had moved two-thirds of the way down the gel. Gels were photographed under UV illumination (365 nm), and a rough estimate of RNA concentration was obtained by comparing the intensity of staining of the sample lane with staining of adjacent lanes containing a known quantity of RNA.

To generate Northern blots the RNA gels were washed three times in milliQ water (15 min each at RT) and the RNA transferred from the gel to a similar sized nitrocellulose membrane (S&S NC BA85, pore size 0.45 µm). Prior to transfer the nitrocellulose (NC) membrane was prepared first by wetting in milliQ water and then soaked in 10 X SSPE (308). To effect transfer the gel was inverted (e.g. wells opening down), placed on an inverted gel tray, and the following items carefully applied to the exposed surface of the gel: 1) the NC membrane soaked in 10 X SSPE, 2) two sheets of 3 MM filter paper cut the same size as the gel, and previously soaked in 20 X SSPE, 3) a stack of paper towels approximately 2.5 inches thick. After placing the various components the whole assembly was 'flipped' so that the gel tray was now on top, and the paper towels on the bottom. Blotting was allowed to continue overnight or longer if convenient, following which the whole assembly was taken apart and the location of the gel wells (now collapsed) were marked on the NC membrane using a black tipped pen. The membrane was left to air dry, and then sandwiched between two sheets of 3MM filter paper and baked at 80°C in a vacuum oven for 2 h.

2. *Preparation of radioactively labeled probes*

a. Origin of plasmids containing mouse kappa and gamma 1 cDNA probes-

The plasmids pJRC45 and pJRC52 contain cDNAs for the mouse kappa and gamma 1 chains respectively (both are cloned as *Eco*RI inserts in pBR322). The plasmids were originally constructed by J. Carlson, and were obtained as a gift from D. Denney (Stanford).

b. Large-scale preparation of plasmid DNA on CsCl /ethidium bromide gradients-

Bacteria containing plasmids were grown in 2xYT media (308), harvested and lysed by the alkali method as described in Molecular Cloning a Laboratory Manual (308). Purification of plasmid DNA was performed by the CsCl-ethidium bromide gradient (308). Ethidium bromide was removed from the DNA by repeated extractions with water-saturated n-butanol (308), the plasmid DNA in the aqueous phase was precipitated by adding 2.5 volumes of TE, sodium acetate (pH 5.2) to Cf 0.3 M, and finally an equal volume of isopropyl alchohol. Precipitation was aided by freezing on dry ice and then thawing completely before spinning at 4°C, for 30 min at 12,000 xg. The DNA pellet was washed with 1 ml of 70% (v/v) ethanol, dried briefly, redissolved in 400 µl of TE, and extracted once with phenol/chloroform and twice with Cevag (24:1 (v/v) chloroform : isoamyl alcohol). The DNA was precipitated by adding sodium acetate (pH 5.2) to Cf 0.3 M, 2.5 volumes of 95% ethanol, and freezing on dry ice. The pellet was washed with 1 ml of 75% ethanol, redissolved in TE, and DNA concentration determined by measuring OD_{260}, using the conversion factor 1.0 A_{260} = 50µg/ml.

c. Generation of hybridization probes by radioactively labeling DNA fragments in LMP agarose gel slices using random hexamer primers

Restriction enzyme digests were performed (e.g. *Eco*RI for pJRC45 which contains the mouse kappa cDNA as an *Eco*RI fragment) and the reaction products separated on a 0.8% LMP agarose gels cast and run in TAE buffer (308). The band

(e.g. DNA fragment) of interest was carefully excised from the gel, melted at 70°C for 3 min and re-loaded 'dry' (e.g. prior to addition of buffer to the gel tank) and run on another 0.8% LMP agarose/TAE gel. The DNA fragments to be labeled were run twice on a gel to ensure that there was no vector contamination, since this might confound the hybridization results. The final band of interest was excised, the total volume of the gel slice estimated, and an equal volume of milliQ water added. The gel slice + water was melted at 70°C, mixed thoroughly, and 33 µl (or all available) of the melted slice was transferred to a fresh Eppendorf tube and then boiled for 7 min to denature the DNA. The aliquot of boiled DNA/gel slice was cooled to RT and the following solutions added: 10 µl OLB, 2 µl of 10 mg/ml BSA, 5 µl of α^{32}P-dCTP (Amersham ; high specific activity), and 5-10 U Klenow enzyme (20 U/µl; NEB). OLB consists of 0.25 M Tris-HCl, 25 mM magnesium chloride, 0.05 M β-mercaptoethanol, 0.1 mM of each of dATP, dGTP and dTTP, 1.0 M Hepes pH 6.6 and 27 A_{260} units/ml of random hexamer oligonucleotides. If less than 33 µl of gel slice was used, the final reaction volume was brought up to 50 µl with milliQ water. The reaction components were mixed thoroughly, incubated at RT for 1- 4 h, and the reaction terminated by adding 5 µl of 10% (w/v) SDS, 5 µl of 0.5 M EDTA pH 8.0, and heating at 70°C for a few minutes. Unincorporated nucleotides were then separated from the labeled DNA fragments by passing the reaction mixture over a small gel exclusion (e.g. 'drip') column. These columns have the advantage that they cannot run dry, and thus can be run with intermittent additions of buffer to the top of the column.

To construct a 'drip' column, a glass Pasteur pipette was plugged with a small piece of sterilized glass wool, and then filled with Sephadex-G50 fine beads which had been pre-swollen in TE. The column was allowed to settle by passing 3 ml of TE through the beads, after which the heated labeling reaction (from the 70°C bath) was loaded immediately onto the column. Once the reaction mixture had entered the column bed, 500 µl of TE was added and allowed to enter the column. Following this a second 500

µl aliquot of TE was added to the column, and 1 drop column fractions began to be collected immediately thereafter. Two additional 500 µl aliquots of TE were added, and column fractions continued to be collected until these volumes had entered the column bed. A rough survey of the radioactivity present in each of the column fractions was carried out using a hand-held radiation monitor. In general there were two separate peaks of radioactivity which eluted from the column, and fractions comprising the first peak were pooled to generate the radioactive probe. To assess the total yield of labeled DNA probe, 1 µl of the combined excluded peak fractions was spotted on a filter paper, dried, and measured in a scintillation counter (with scintillation fluid) on the ^{32}P channel. The probe was either used directly or it was frozen at -20°C for later use. In some cases a 1 ml disposable plastic syringe barrel was used in place of a Pasteur pipette to construct the drip column, in this case a small disc of filter paper was used to plug the end of the column, and it was run as above, with some adjustments to the volumes used.

3. *Probing and washing of northern blots*

The dried, baked Northern blot was soaked in 4 X SSPE, then transferred to a container with prehybridization solution (50% (v/v) formamide, 200 µg/ml salmon sperm DNA, 5 X SSPE, 0.1% SDS and 5 X Denhardt's solution; (308)). Prehybridization was performed at 37°C for 0.5-4 h on with constant slow shaking.

The hybridization solution was the same as for prehybridization. However, for the purpose of denaturing the probe, the probe was first added to the desired volume of pure formamide, after which the rest of the hybridization-solution components were added. Alternately the probe solution was boiled for 5 min prior to addition to the complete hybridization solution. Hybridizations typically were continued overnight at 37°C with constant gentle shaking.

Following pre-hybridization and hybridization the membranes were washed 2-3 min without shaking, using a generous volume (e.g. 500 ml) of 2 X SSPE, 0.1% (w/v) SDS prewarmed to RT. Two additional 10 min washes were carried out with gentle

shaking at RT using the same buffer and volumes as for the first wash. Depending on the strength of the signal (as determined by using a hand-held monitor) 2-3 additional washes of 10 min each were carried out in 0.2 X SSPE, 0.1% SDS at 56°C, again with continuous gentle shaking. Washing was continued until the background areas of the filters showed a low signal, and the membrane was air dried briefly, wrapped in Saran Warp, and exposed to X-ray film using an intensifying screen.

In cases where membranes were re-hybridized to a second probe, they were first 'stripped' of the existing probe by placing them in boiling milliQ water for a few min. The stripped membranes were checked with a hand-held monitor and confirmed by exposure to X-ray film prior to hybidization to the second probe.

C. Construction of an improved lambda-based cDNA cloning vector: λZapII*Sfi* IB

In 1987 (309) Aruffo and Seed created a plasmid vector called pCDM8 (B. Seed, personal communication), which has two special *Bst*XI restriction sites (general recognition sequence CCANNNNNNTGG) separated by a 360 bp 'stuffer' fragment (figure 9). When pCDM8 is digested to completion with *Bst*XI and the vector fragment separated from the stuffer, both ends of the prepared vector have the identical 3' overhanging 'sticky' end; in this case -TGTG. Since the overhanging ends cannot anneal to each other, it is impossible for the vector to re-circularize (these ends can be classified as 'symmetrical but non-self cohesive'). In fact the vector can only be circularized by re-inserting the 'stuffer' fragment, or by inserting any other fragment of DNA to which has been ligated the '*Bst*XI linker-adapters' shown in figure 9. This type of cloning strategy is extremely efficient for constructing cDNA libraries, since the vector does not need to be dephosphorylated (and thus it can be prepared very easily), and the identical linker-adapter can be ligated very simply to either end of the blunt ended, double-stranded cDNA without requiring any further digestion of the cDNA prior to ligation into the vector.

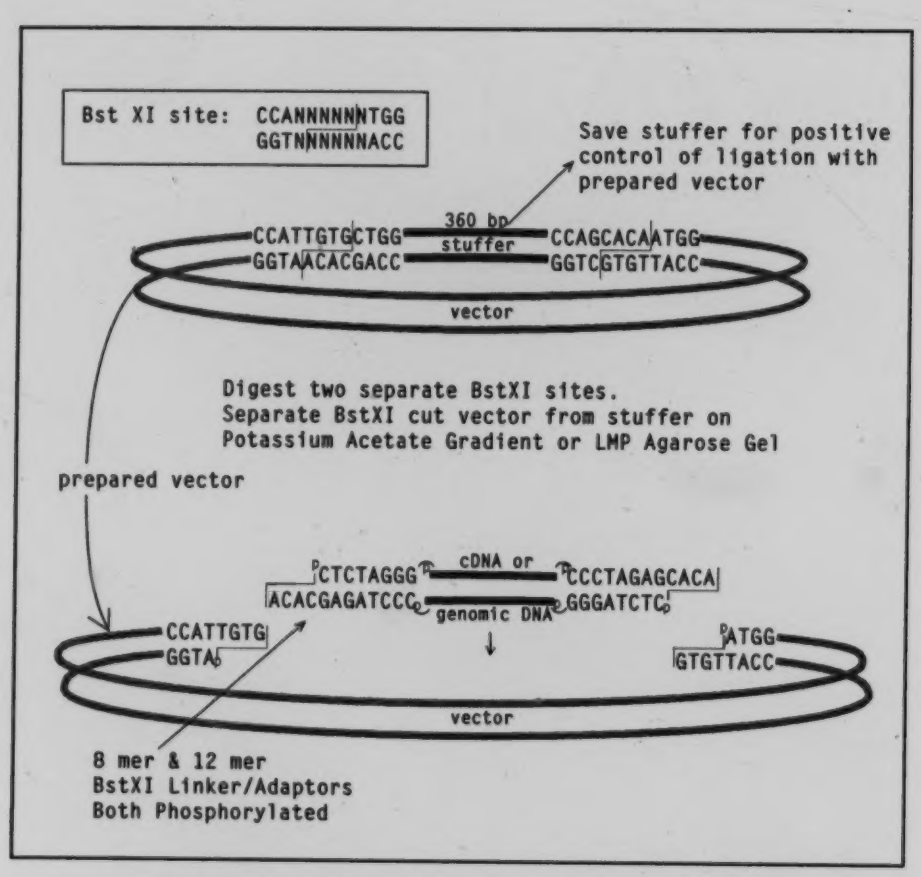


Figure. 9 Generation of a vector with symmetrical but non self-cohesive ends, using *Bst*XI (e.g. pCDM8, after Brian Seed). Identical linker/adaptors are ligated on each end of the blunted cDNA or genomic DNA, and after size selection these are efficiently ligated into the prepared plasmid vector. As in the previous figure, the linker/adaptors can ligate with each other, and these must be removed by spermine precipitation and size selection.

The objective of constructing λZapII*Sfi* IB was to create a lambda based cDNA cloning vector which would make use of the same type of high efficiency cloning strategy which had been achieved using pCDM8. Lambda vectors have the advantage that highly efficient in vitro packaging can be used to create the primary clones, and also that once plated, the resulting libraries are much easier to screen and much more stable than are plasmid libraries. Since the lambda genome contains many *Bst*XI sites, a new restriction site was required to carry this approach into lambda-based vectors. We chose to use *Sfi*I (GGCCNNNNNGGCC) since this 8-base recognition sequence is absent from the lambda genome, and as with *Bst*XI, the ambiguous sequence in the centre made it possible to generate 'symmetrical but non-self-cohesive' ends (e.g. GTG; figure 10). This vector thus makes it possible to realize the very high cloning efficiency of pCDM8, but in a lambda vector.

1. Preparation of the *Sfi* IB stuffer

As shown in figure 11 the two oligos were phosphorylated and annealed. The original oligo stock was diluted to 4 A/100 µl, and a 100 µl of this was used in a 200 µl phosphorylation reaction. The 200 µl also contained 20 µl of each of 10 X ligase *Sal* ts (500 mM Tris-˙HCl pH8.0, 100 mM $MgCl_2$) and of 10 X ATP/DTT (10 mM ATP/100 mM DTT), and of 10 µl of Kinase (Biolabs). The reaction was incubated at 37°C for 2.5 h. Annealing of the phosphorylated oligos was carried out by mixing 22 µl of '2*Sfi*I-UP' with 14 µl of '2 *Sfi*I-DN' (for equamolar amounts). The reaction was incubated at 70°C for 10 min followed by a 15 min incubation at 37°C. The 389 bp DNA piece was obtained by digesting 4 µg of pJFE14 (see figure 12) (310) with *Bst*XI, followed by 1 phenol/cevag and 2 Cevag extractions. The DNA was precipitated using 3 volumes of 95% ethanol in the presence of 0.3 M sodium acetate (pH 5.2) and was further purified by a spermine precipitation.

a. Spermine precipitation

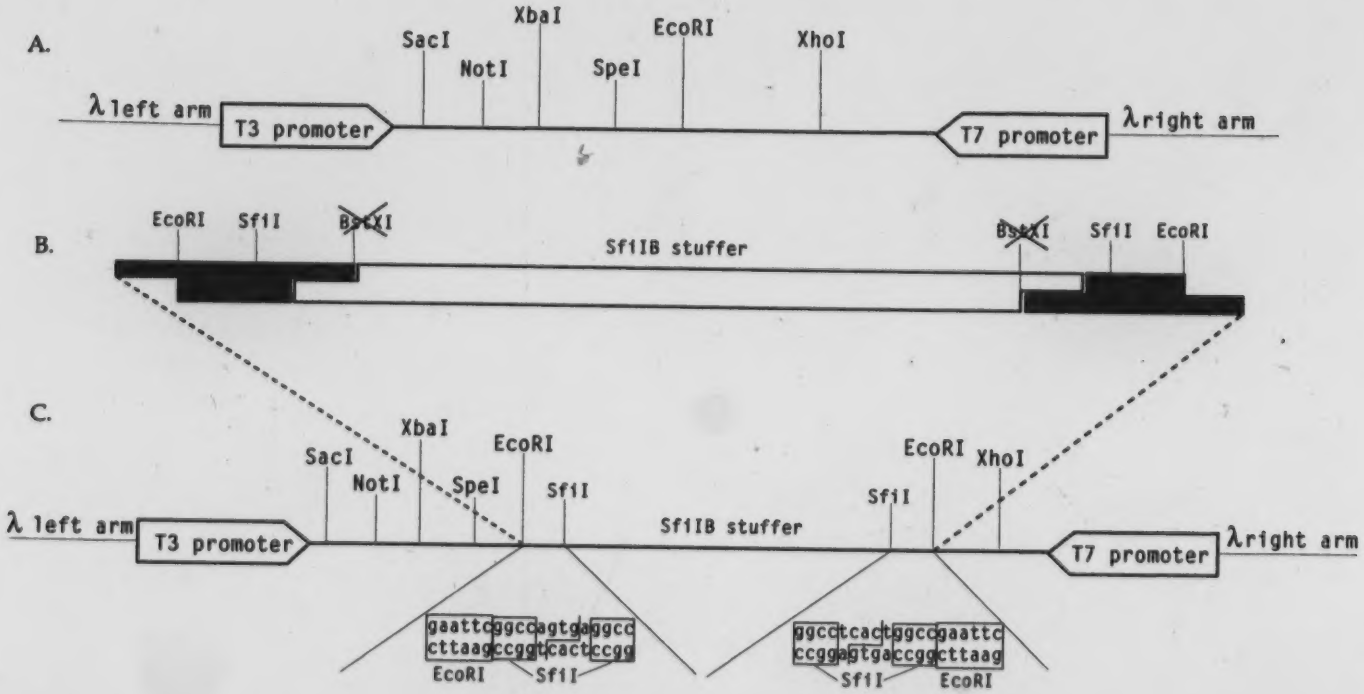


Figure 10. λZapII*Sfi*IB. A. Unique restriction sites in λZapII polylinker. B. *Sfi*IB stuffer (see figure 11). C. λ ZapII *Sfi*IB. (confirmed by sequencing.)

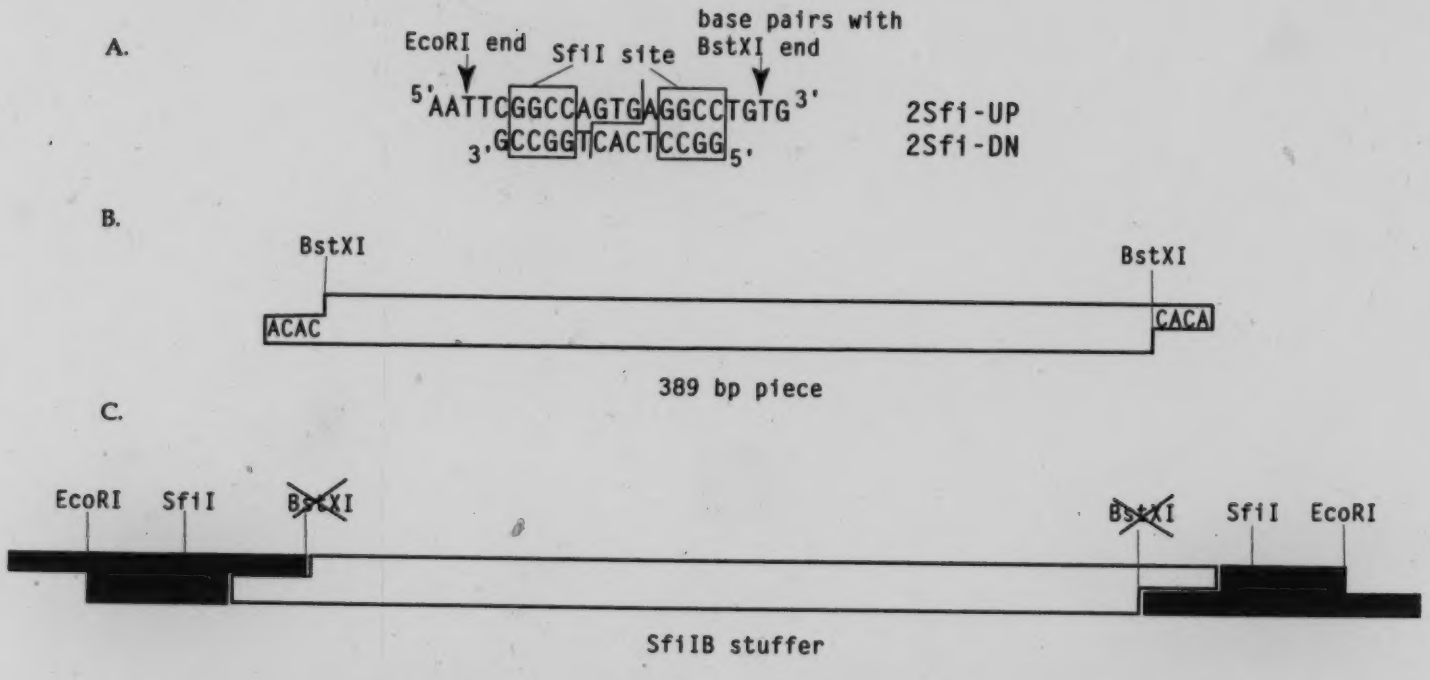


Figure 11. Steps in the construction of Lambda ZapII*Sfi*IB. A. Phosphorylated and annealed linker-adaptors with one *Bst*XI "sticky" end and one *Eco*RI end. B. Brian Seed's 389 bp "stuffer" piece digested with *Bst*XI. C. The final *Sfi*IB stuffer, generated by ligating a 20 fold molar exess of the linker adaptors to the *Bst*XI cut stuffer, digesting with *Eco*RI, and cloning into *Eco*RI digested, dephosphorylated pBluescript SK⁻. The *Eco*RI insert from this plasmid was then excised and ligated into *Eco*RI cut, dephosphorylated λZAPII. (See previous figure).

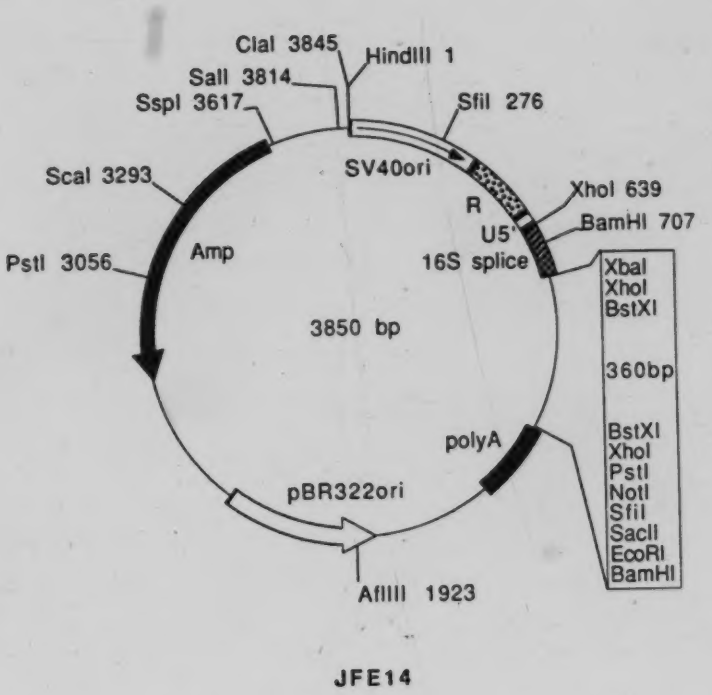


Figure 12. Map of pJFE14. After Elliott et al (310). SV40, ori, R and U5' are from pSRα

The DNA pellet was redissolved in 50 µl of TE. Two µl of 100 mM spermine was added, mixed thoroughly and incubated on ice for 15-20 min with occasional vortexing. DNA was pelleted in a microfuge (15-20 min at 16,000 xg, at 4°C), and 1 ml of extraction buffer (75% ethanol, 0.3 M sodium acetate and 10 mM magnesium acetate was added to the pellet followed by a 15-50 min. incubation on ice. DNA was pelleted as above and the pellet was rinsed with 1 ml of 75% ethanol.

b. Ligation of *Sfi*I adapters (creation of the *Sfi*IB stuffer)

Half of the amount of DNA recovered from the spermine precipitation was used in a ligation reaction with 3 µg of the annealed phosphorylated oligos and 40 units of ligase in a total reaction volume of 45 µl. The reaction continued overnight at RT and was terminated by heating to 70°C for 20 min. The reaction was then run on a LMP agarose gel to remove unligated linkers and the *Sfi*IB stuffer band, with the ligated *Sfi* I linkers, was excised from the gel to be cloned into pBluescript SK$^-$.

2. Cloning the *Sfi* IB stuffer into λZapII

To obtain a reasonable amount of purified preparation of the *Sfi* IB stuffer to be cloned into λ Zap II, I cloned the stuffer into *Eco*RI cut pBluescript SK$^-$ first (the resulting plasmid was SK$^-$*Sfi* IB). The SK-*Sfi* I B plasmid was then used to transform *E. coli* DH5α cells, a plasmid preparation was made and the *Sfi* IB stuffer was digested out with *Eco*RI and cloned into *Eco*RI cut, dephosphorylated λ Zap II . The *Sfi* IB stuffer band in the LMP gel slice (from above) was melted at 70°C for 3 min, and 5 µl was taken out and used in a ligation reaction (Vᴛ 40 µl, same as above) to 50 ng of SK$^-$ *Eco*RI digested and dephosphorylated vector. Five µl of the ligation reaction was used to transform 100 µl of frozen competent DH5α cells. After at least 14 h, colonies were picked and grown overnight in 2 ml of 2xYT with the appropriate antibiotic. Plasmid DNA was then isolated by the alkaline lysis minipreparation method, or by the Triton lysis minipreparation procedure. The plasmids were mapped for the correct insert by means of restriction digests.

a. Preparation of calcium chloride competent cells

A 500 ml culture of bacteria (e.g. DH5α) grown in 2xYT/MT to an OD_{600} of 0.5 was chilled on ice for 10 min. Cells were pelleted at 2,000 xg for 10 min at 4°C. The pellet was resuspended in 0.5 of the original volume with chilled 50 mM $CaCl_2$/20 mM Hepes and pelleted again. Cells were resuspended this time in 1/10 of the original volume of chilled 50 mM $CaCl_2$/20 mM Hepes + 5% (v/v) glycerol. The suspension was aliquoted into prechilled tubes and frozen on powdered dry ice.

b. Heat shock transformation of competent cells

Five µl of a ligation reaction was mixed with thawed competent cells (50 µl of *E. coli* TG2 cells, needed for single stranded (ss) DNA preparation for sequencing, or 100 µl of *E. coli* DH5α cells) and incubated on ice for 20-40 min. The DNA/cell mixture was heat shocked at 42°C for 90 s, incubated on ice for 3-5 min and 1 ml of 2xYT (with no antibiotics) was added and the cells were left to recover on the roller wheel for 40-90 min at 37°C. Two concentrations of transformed cells were plated; a 100 µl of the culture and the whole cell pellet. Cells were pelleted by pouring the culture into an Eppendorf tube and centrifuging in a microfuge at 16,000 xg for 5 s. Following outgrowth cells were plated on 2xYT plates containing the appropriate antibiotics.

c. Alkaline and Triton lysis plasmid minipreparation

Alkaline lysis was done following Molecular Cloning a Laboratory Manual (308) with the optional one phenol and one Cevag extractions. Lysis by boiling (Triton lysis) (308) was modified by boiling for 2 min.

Shown in figure 10 is a schematic representation of the construction of λ ZapII*Sfi* IB. SK-*Sfi* IB (4 µg) was digested with *Eco*RI to obtain the *Sfi* IB stuffer for cloning into λ ZapII. The digest was run on a 0.8% LMP agarose gel, the stuffer band was cut out and eluted using a "freeze squeeze" method. The agarose slice was frozen on dry ice for 15 min and centrifuged in a microfuge for 30 min at 16,000 xg at 4°C and DNA was recovered in the liquid. Two µl of this was used in a 10 µl ligation reaction with 2

µl (about a 100 ng) of *Eco*RI digested and dephosphorylated λ ZapII. The ligation reaction was incubated overnight at 14°C, and 2 µl of the ligation reaction was then packaged with home-made packaging mixture of freeze-thaw and sonic extracts and plated. Nitrocellulose membranes were laid down on the phage plates, removed (308), and screened with the 389 base pair 'stuffer' (radiolabeled with ^{32}P dCTP). Hybridizing plaques were isolated, *in vivo* subcloned (e.g. 'zapped out') and sequenced.

d. Packaging and plating of λZapII*Sfi* IB

Home-made packaging extracts were used. When packaging phage DNA two types of extracts are needed, freeze thaw and sonic. The freeze thaw provide the proteins needed for packaging and the sonic extract provides the phage proheads.

The freeze thaw and sonic extracts were prepared as in Molecular Cloning a Laboratory Manual (308). However, instead of incubating the culture in a 45°C water bath for induction, to raise the temp. quickly to 45°C, the flask was heated (while swirling) on a Bunsen burner with an ethanol-wiped thermometer in it.

Two µl of the ligation reaction was added to a thawed aliquot of freeze-thaw extract, followed by the addition of 45 µl of the thawed sonic extract, the contents were mixed by flicking and incubated at RT for 1 h to package.

To plate out the phage, 1 ml of SM media (308)was added to the packaging mixture, mixed, and serial dilutions in SM medium were made. To prepare the bacterial plating stock, 50 ml of 2xYT/MT was inoculated with an overnight culture of *E. coli* BB4, maltose was added to a final concentration of 0.2%, and after 6-7 h (just before plating) $MgCl_2$ was added to a final concentration of 10 mM.

For each dilution of the packaging mixture, a sterile glass tube was prepared by mixing four drops of *E. coli* BB4 culture (or 0.7 ml if using large plates) with 100 µl of the appropriate phage dilution in SM and incubating at RT for 20 min. To pour the plates, 1.5% agar-containing 2xYT/MT plates were taken out of the cold room a day before and incubated at RT so that the top agar, 0.75% agar in 2xYT/MT (would adhere

to the 1.5% agar surface on the plate). Three ml of liquified top agar (equilibrated at 47°C) were added to each of the dilution tubes, mixed by rolling the tube between both hands so as not to introduce air bubbles, and poured onto the plate surface. After solidification the plates were incubated inverted at 37°C to obtain plaques, after which they were kept in the cold room.

e. Screening for recombinant phages

Millipore nitrocellulose filters (HA, pore size 0.45 µm) were marked using a ball point pen, with information to identify the plate, and placed on the agar surface of a prechilled plate containing phage plaques for 2 min. Using a drawn out Pasteur pipette dipped in India ink (Sanford Inc.), holes were made through the membrane and into the agar to mark the position of the filter relative to the plate. The filters were peeled off with Millipore forceps and bathed (DNA side down) in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 40 s, then in neutralizing solution (1M Tris-HCl pH 8.0, 1.5 M NaCl) for 2-3 min. Membranes were blotted to dry on filter paper (DNA side up), sandwiched between two 3MM filter papers and baked in a 80°C vacuum oven for 2 h.

See the section on northern blots for details on prehybridization and hybridization. Both hybridization and prehybridization solutions were pre-warmed to 65°C before adding the filters. Hybridization was overnight at 65°C, in a waterbath shaker (New Brunswick Scientific Model G76D, USA, Gyrotory) set at 90 rpm.

The filters were washed (in 0.05% SDS, 0.2X SSPE) 3 times (in a 55°C water bath, 132 rpm, for 15 min/ wash) with the fourth wash being at 65°C, if necessary. Filters were taped onto a film-size piece of 3MM paper. To be able to orient the film (after developing), the 3MM paper was marked with 'hot' ink (India ink mixed with old stocks of ^{32}P). Membranes were wrapped with Saran Wrap and exposed to X-ray film for 1.5 h.

Agar plugs containing plaques hybridizing to the probe were cut out using the narrow end of a Pasteur pipette. Phage was eluted out from the agar plug by placing the

plugs in an Eppendorf tube with 1 ml of SM solution (308)(with a drop of chloroform) and vortexing frequently over a period of a few hours (or left overnight) .

f. In vivo excision (Zapping) and sequencing of the recombinant phage

To sequence positive λ clones to see if they contained the '*Sfi* IB stuffer', the SK-plasmid which resides within the phage had to be 'zapped' out (*in vivo* excision). In a glass culture tube, 100 µl of the eluted virus (from the agar plug) was mixed with 3 drops of the bacterial strain TG2 (grown and prepared in the same way as was done for BB4 plating stock), and 40 µl of helper phage R408 (~5XI0^{11} pfu/ml) was added. The tube was incubated in a 37°C water bath for 20 min. Two ml of 2xYT/MT were added to the phage-bacterial mixture and the culture was incubated overnight at 37°C on a tube roller. Bacterial cells were pelleted by centrifugation in a microfuge at 16,000 xg at 4°C for 7 min. Culture supernatant was taken off and heated for 20 min at 70°C, resulting in transducing particles (tps) which could be stored at 4°C for many months. To isolate the plasmid, 300 µl of a thick, fresh overnight culture of TG2 was mixed with 30 µl of tps and incubated at 37°C for 20 min. Bacteria were then streaked on LB/Amp plates and incubated at 37°C. Fresh colonies were picked (large colonies only), plasmid mini-preparations were made, and plasmids were mapped with restriction enzymes (see previous section). DNA from one of the positive clones was sequenced using the dideoxy Sequenase kit (US Biochemicals) and α[^{35}S] dCTPs labeling.

3. Preparation of λZapII*Sfi* IB arms

A large scale DNA preparation of λZapII*Sfi* IB was isolated and digested with *Sfi* I. λZapII*Sfi* IB arms were then separated from the stuffer by ultra centrifugation on potassium acetate gradients.

a. Large scale isolation of λZapII*Sfi* IB DNA

Agar plugs containing plaques hybridizing to the probe were isolated and recombinant phages were eluted into SM medium ,as above, and phage concentration was determined by titration. Between 20-40 plates (150 x 15mm) were plated (see

previous section on plating) with an expected density of $1XI0^5$ pfu/plate. To obtain a dense phage infecting stock, we either washed one of the plates that showed confluent lysis (prepared in the previous section) with 5 ml of SM and then quantified by titration, or we used 200 µl of the original SM elute (from an eluted individual plaque in an agar plug) with 200 µl of BB4 and incubated to infect at RT for 15 min, then added 2 ml of 2xYT/MT and incubated overnight until cells were lysed. The lysed culture was then titred and used as the infecting stock.

After plating, plates were incubated overnight until confluent lysis was observed. Phage were washed off the plates by adding 12.5 ml/plate of lambda dilution medium (10 mM Tris-HCl pH, 10 mM $MgCl_2$) and incubating the plates on a shaking platform at 4°C overnight (24-36 h preferred). Eluted phages were pooled into 250 ml bottles and agar and bacterial cells were pelleted by centrifugation at 8K for 30 min at 17°C in a JA14 rotor. Another centrifugation was performed to pellet the phage (25000 rpm [~ 28000 rpm = 141,000 xg], 4°C for 75 min using the SW28 rotor). The well drained pellets were resuspended in "lambda dillution medium" and pooled to a final volume of 35 ml and banded 3 times on CsCl gradients. For the first phage spin, 3 ml of CsCl at 1.45 g/cc (59 g CsCl/100 ml of lambda dil) was added to each of the SW40.1 tubes; this was underlaid with one full Pasteur pipette of CsCl at 1.50 g/cc (69 g CsCl/100 ml of lambda dil). Equal amounts of phage elutes were slowly overlaid on top of the CsCl solution in each of the tubes. The tubes were then filled and balanced with "lambda dil" and centrifuged at 28000 rpm in a SW 40.1 rotor (25000 rpm = 111,000 xg), at 20°C for 1.5 h. The blue phage bands were found between the 2 CsCl densities, and were collected with an 18 gauge needle. The second spin was performed by first adding 0.6 g of CsCl to every ml of phage suspention. This was mixed and then transferred to SW40 tubes. The phage layer was covered with 5 ml of CsCl at 1.50 g/cc followed by 3 ml of CsCl at 1.45 g/cc. The gradient was centrifuged and bands were removed as above. The phage bands were pooled and two volumes of "lambda dil" were added and

a third spin was done exactly like the first one . The bands were pooled and 1/10 volume of 2 M Tris-HCl pH8.0 , 1/25 volume of 0.5 M EDTA, 1/10 volume of 10% SDS and 9 volumes of 100 mM NaCl were added and mixed. The mixture was extracted twice with phenol/Cevag, twice with Cevag and back extracted with 100 mM NaCl. Phage DNA was then precipitated with 95% Ethanol and a 30 min incubation on ice. The DNA threads were spooled out and washed twice in 75% ethanol on ice (each wash for 15 min). The final wash was done in 95% ethanol and the pellet was air dried for 5 min at RT and then redissolved in 400 ml of TE.

b. Separation of λZapII*Sfi* IB arms from the *Sfi* IB stuffer

Purified λ DNA was digested overnight at 56°C using *Sfi* I (8 units/μg DNA), and digests were loaded directly on a 5-20% potassium acetate gradient made in 2 mM EDTA, 0.1 μg/ml Ethidium bromide formed in 1/2 x 2 clear ultracentrifuge tubes. Tubes were spun in an SW 55Ti rotor at 304,000 xg for 30 min, and λ bands were removed using an 18G needle. The DNA was extracted twice with water-saturated n-butanol, precipitated with sodium acetate/ethanol, and redissolved in TE.

D. The construction of B [PK3B] and PK99H cDNA libraries in λZapII*Sfi*IB

First and second strand cDNA synthesis reactions were performed and were followed by a spermine precipitation. The 5' and 3' ends were made blunt with Klenow and the cDNA was purified by 1:1 phenol:Cevag extractions and ethanol and then spermine precipitations. *Sfi* I linkers were ligated on to the ends of the cDNA molecules. The products of the ligation reaction were run on an agarose gel for size selection and to remove unligated linkers. The cDNA was extracted from the agarose gel slice with β-agarase and subjected to another spermine precipitation. λZapII*Sfi* IB arms were used in a ligation reaction with the size-selected and purified cDNA, followed by packaging and plating of the B and PK99H cDNA libraries.

1. First strand cDNA Synthesis

First strand cDNA was reverse transcribed from 25-30 µg of PK99H total RNA and 10 µg of B poly A+ RNA (purified using the Promega Poly A isolation kit). cDNA synthesis was performed in a 100 µl total volume. The volume was adjusted using sterile milliQ water containing 20 units/50µl RNasin (Promega). The reaction contained 10 µl of 10 X reverse transcriptase buffer (0.5 M Tris-HCl pH 8.2, 0.5 M KCl, 60 mM $MgCl_2$), 10 µl of oligo dT (Collaborative Research Labs Inc.; 1 mg/ml), 4 µl of 25 mM dNTPs, 1 µl of 1 M DTT. This was mixed well and then 3 µl of AMVRT (Avian myeloblastosis virus reverse transcriptase, Molecular Genetics Resources, Tampa) was added. The reaction was incubated at 42°C for 90 min, followed by a second addition of 2 more µl of AMVRT, with a further 60 min incubation at 42°C. The reaction was then terminated by heating to 70°C for 10 min.

2. Second strand cDNA synthesis

The reaction was carried out in a 500 µl total volume and the following solutions were added: 50 µl of DNA polymerase buffer (150 mM Tris-HCl pH 7.0, 30 mM $MgCl_2$ and 500 mM KCl), 5 µl of 1 M DTT, 2 µl of BSA (10 mg/ml), 2 µl RNase H (Promega) and 3 µl of *E. coli* DNA polymerase (Biolabs), and autoclaved milliQ water to a total volume of 100 µl. In order to label the second strand cDNA (for the B library only), 5 µl of α [^{32}P] dCTP was also added. The reaction was incubated at 14°C overnight, and then at 22°C for 2-4 h.

3. Blunting of cDNA and Ligation of linkers

Before adding the linkers, the ends of the cDNA molecules had to be made blunt using Klenow. The dried pellet (from spermine precipitation of the second strand reaction, done as above) was dissolved in 85 µl of milliQ water, centrifuged in a microfuge at top speed at RT for 5 min and the supernatant was removed to a siliconized tube. 10 µl of 10 X Klenow buffer (NEB) was added followed by 1 µl of 1 M DTT, 1 µl of 25 mM dNTPs and finally 2 µl of Klenow (NEB). The reaction was mixed well and incubated at 37°C for 1-2 h.

To purify the DNA before adding the linkers, a 1:1 phenol:Cevag and two Cevag extractions were carried out (with back extractions of 100 µl of TE). The cDNA was reprecipitated by adding 200 µl of 5 M ammonium acetate, 5 µl of 100 X linear polyacrylamide (250µg/ml polymerized with no bis-acrylamide) and the tube was filled up with 95% ethanol. The cDNA was washed as in previous sections. The cDNA pellet was then dissolved in 100 µl of 0.1 M KCl and spermine precipitated.

To ligate the linkers, the cDNA was dissolved in 28 µl of TE followed by the addition of 1.5 µl of the phosphorelated 8 mer (Blunt *Sfi* IB), 3.5 µl of the phosphorelated 11mer (Blunt *Sfi* IB, for sequence see table 3), 2µl of 10 mg/ml BSA, 4 µl of NEB 10X T4 DNA ligase buffer and 1.5 µl of ligase (40 unit/µl), mixed and incubated at 14°C overnight.

4. Size selection of the cDNA fragments

The ligation reaction was run on a 1% LMP TBE gel for size selection and for elimination of the unligated linkers. The gel ran for 30-40 min at 60 volts. cDNA ranging in size from 1-2 Kb was cut out and extracted from the agarose with the use of β-agarase (NEB) according to the manufacturer's instructions.

5. Ligation to λ ZapII*Sfi* IB arms

The PK99H cDNA concentration was measured with the use of a florometer and 52 ng of cDNA was ligated to 400 ng of λ ZapII*Sfi* IB arms. The B cDNA concentration was not measured, however, two dilutions were tested; see below. NEB T4 DNA ligase buffer was used in a final volume of 10 µl with 0.4 µl of ligase; the reaction was incubated at 14°C overnight.

6. Packaging and Plating

For detailed procedure see the previous section. The cDNA was packaged first with home-made packaging extracts and based on the plating results, 3 µl of B cDNA (for one of the two tested dilutions) was packaged with Giga gold kit (PDI, Biosciences, Stratagene) for 1 h at RT. The Giga packaged B library was plated on 10 large (150 x

15mm) plates (expected density of 30,000 plaques/plate) , and the home-made extract packaged PK99H library on 2 (150 x 15mm) plates (expected density of 33,000 plaques/plate). For the actual results see table 5.

E. The isolation of V_H and V_L sequences by screening B [PK3B] and PK99H cDNA libraries with V and C-region probes

1. Preparation and labeling of constant and V-region sequence specific probes

The N-terminal end of the heavy and light chain proteins (for PK99H and only the light chain for B) were sequenced and based on this sequence 5' degenerate primers were synthesized and used in combination with constant region primers to amplify V-region sequences. For rationale see chapter 3.

First strand cDNA was synthesized from B and PK99H as was done in the construction of the cDNA libraries, except that 1 µl of 1 µg/µl of oligo dT was used instead of 10 µl and no second addition of AMVSRT was performed. Two rounds of amplification reactions using the degenerate 5' primers in combination with constant region primers were carried out and the amplified cDNA was cleaned and ends were blunted using T4 polymerase. 'Has-Link 12' and '2Sfi-UP' (table 3) adapters were phosphorylated and ligated to the blunt-ended cDNA, which was then ligated to *Bst*XI digested pHAS (see figure 40). Ligation reactions were then used to transform *E. coli* DH5α cells, colonies were picked, plasmid DNA preparations were made and clones were mapped to determine whether they contained the amplified insert by either restriction digest mapping or by RT-PCR amplification. Plasmids containing amplified inserts were then used to transform TG2 cells to obtain single stranded DNA which was later used in ^{35}S-dCTP sequencing reactions (311) with T3 primer. Sequencing was carried out following "Protocol For DNA Sequencing With Sequenase version 2.0" (USB, Cleveland, Ohio). Sequencing was also done, again using the T3 primer, with fluorescent-dye-labeled terminators on an ABI machine (model 373).

Table 3. Oligonucleotides used as linkers/adaptors and those used in I-RT-PCR reactions

Linkers/Adaptors:	
Has-link 12	CCCTAGAGACAC
Has-link 8	CTCTAGGG
2Sfi-UP	AATTCGGCCAATGAGGCCTGTG
2 Sfi-DN	GGCCTCACTGGCCG
Blunt SfiIB 8	CTCTAGGG
Blunt Sfi1B 11	CCCTAGAGCAC
Leader B/S-DN	ATGGTGGGTCGACAGAGA
Leader B/S-UP	CTCTCTGTCGACCCACCATGCCC
H/E-UP	AGCTTAGAACTG
H/E-DN	AATTCAGTTCTA
Sph1/EcoR1 8	CAAGCTTC
Sph1/EcoR1 16	AATTGAAGCTTGCATG
I-RT-PCR:	
Kc5'(IPCR)P/E2	TCTCTCTGCAGAATTCGATACAGTTGGTGCAGCATC
Kc3'(IPCR)aX/S	TCTCTCTCGAGTCGACAAGAGCTTCAACAGGAATGAG
Kc5'(IPCR)a	CACACGACTGAGGCACCTCCAGATG
Kc5'(IPCR)	AGATGTTAACTGCTCACTGGATGG
Kc3'(IPCR)a2	GCCACTCACAAGACATCAAC
Kc3'(IPCR)a	AAGAGCTTCAACAGGAATGAG
µc5'(IPCR)P/E	TCTCTCTGCAGAATTCGACATTTGGGAAGGACTGAC
µc3'(IPCR)X/S	TCTCTCTCGAGTCGACCATGTCTGACACAGGCGGC
µc5'(IPCR)b	GACAGGGGGCTCTCGCAGGAGAC
µc3'(IPCR)b	GAGGACCGTGGACAAGTCCAC

Constant region probes were amplified to be used in screening the libraries to detect those chains where no V-region sequence specific probes were available. Both C-region amplified bands and restriction digests of Qiagen midi plasmid preparations of 8a5 and 126 plasmid DNA were run twice on LMP TBE agarose (to isolate the V-region sequence specific probes). The bands were cut, melted and labeled as in previous sections, except that this time, two to three labeling reactions were done for each probe to increase the amount of labeled probe.

a. The isolation and cloning of V-region sequence-specific probes

i. Amplification of V-region sequence-specific probes

The following combinations of primers were used (See table 4 for sequence and figure 13): 3' and 5' γ actin primers to check for the quality of the templates, for PK99H light chain the 'K5' constant' was used in combination with 3 different primers; '99hKV1', '99HKV2' and '99HKV3'. For PK99H heavy chain, 'IgG1 5' constant' was used in combination with 'IgG1 99H HV1'. For B light chain, 'K5' constant' was used in combination with '3BKV1'.

Five µl of 10 X Taq buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl pH8.3) was added to the 5 µl of ss cDNA, this was followed by 2.5 µl of 5 mM dNTPs, 1 µl of each primer (50 µM stocks) and the volume was made up to 50 µl with sterile milliQ water. The components were mixed and finally 0.5 µl of Taq (home-made) was added. The reaction was allowed to run for 30 cycles with 1 min at each of 94, 72 and 50°C in a home-made cycler system.

The amplification reactions were run on a 1.3% TAE agarose, the correct- size bands were cut out and DNA was extracted from the agarose with the use of the gene clean kit (gene cleaned) (Bio 101 inc. La Jolla) following manufacturer's instruction. Extracted DNA representing each of the isolated bands were used as templates in a second round of

Table 4. Oligonucleotides used in all Conventional-RT-PCR reactions

5' Primers:	
99H KV2	AT(T/A)CT(G/A)GCTGT(G/T)CT(A/T)GC(A/T)GG
99H KV3	AT(T/A)TT(G/A)GCTGT(G/T)TT(A/T)GC(A/T)GG
IgGI 99H HV1	CAGTCTGAGGT(G/A)ATGCT(G/T)GT
5'G actin	CACGCCATCTTGCGTCTG
3BKV1	AT(C/T)CAGATGAC(A/C/T)CAGAC(T/A)AT
K5'	ATCCATCTTCCCACCATCCA
IgG1/3-5'C	CCCCATCTGTCTATCC
YPBstX1 5'	CTCTCCATGCCCATGGGGTCTC
3B5' K-2	CTCTCTGCAGATATCCAGATGACACAGACTATA
3B5' G-2	CGTGCTCGAGTCTTCTCTCTGCAGAGGTCCAACTGCAGCAGCCT
3BK5' Bam	CGCTGGATCCCTCGGAGATATCCAGATGACACAGACTA
HKC5'-XhoI	TCAGGAACCTCGAGTGCACCATCTGTC
3BG5'Bam	CGCTGGATCCCTCGGACAGGTCCAACTGCAGCAGCCTG
EXOμ5' Bam	CGCTGGATCCCTCGGACAGGTTCAGCTCCAGCAGTCTG
EXOK5' Bam	CGCTGGATCCCTCGGAGATATTGTGATGACCCAAACTCCAC
CDμ5' Bam	CGCTGGATCCCTCGGACAAGTGCAGCTGGTGGAGTCTG
CDK' Bam	CGCTGGATCCCTCGGAGACATCAAGATGACCCAGTCTC
Perμ5' Bam	CGCTGGATCCCTCGGACAGGTCCAGCTGCAGCAGTCTG
PerK5' BglII	CGCTAGATCTCTCGGAGATATTGTGCTGACCCAATCTCC
YPO4 NotI 5'	CTCTCGCGGCCGCTAGCCCATGGGGTCTCTGCAAC
T7	TAATACGACTCACTATAGGG
3' Primers:	
K5' constant	GGATACAGTTGGTGCAGCAT
IgG1 5' constant	AGATGGGGGTGTCGTTTTGG
3'G actin	ATTTGCGGTGGACGATGG
PerV3' sal	CTCTCGTCGACGCTGAGGAGACGGTGACTGAG
ExoV3' sal	CTCTCGTCGACGCTGAGGAGACGGTGACTGAG
K3'	ATGTCGTTCATACTCGTCCTTGG
IgGI-3'C	GCTGGAGGGTACAGTCAC
3' leader Bam	CTCTAAGCTTAGTACTCACCTCCGAGGGATCCAGCGACCAG
3B3' K-3	AGAGGCGGCCGCACTTACGCTTGATTTCCAGCTTGGTGCC
3B3' G1-3	AGAGGCGGCCGCACTTACCTGAGGAGACTGTGAGAGTGG
P+E3' K	AGAGGCGGCCGCACTTACGTTTGATTTCCAGCTTGGTGCC
P+E 3'μ	AGAGGCGGCCGCACTTACCTGAGGAGACGGTGACTGAGGT
CDK3'-2	AGAGGCGGCCGCACTTACGTTTTATTTCCAGCTTGGTCCC
CDμ3'-2	AGAGGCGGCCGCACTTACCTGCAGAGACAGTGACCAGAGT
HKC3'-EcoRI	CTCTCGAATTCCTAACACTCTCCCC
CDV3'-SalI	CTCTCGTCGACGCTGCAGAGACAGTGACCAG
CDKV3'-XhoI	CTCTCCTCGAGGTTCGTTTTATTTCCAGCTTGG
3BV3' XhoI	CTCTCCTCGAGGCTGAGGAGACTGTGAGAGTG
T3	ATTAACCCTCACTAAAGGGA

amplifications to reaffirm the identity of the isolated bands and to obtain more DNA to simplify the cloning process. Amplification reactions were once more run on the gel and 'gene cleaned' under the same above conditions. The amplified cDNA was further purified by 1:1 phenol:Cevag, Cevag extractions, ethanol precipitated in the presence of 0.3 M sodium acetate (pH5.2) and rinsed with 70% ethanol.

ii. T4 DNA polymerase blunting of amplified cDNA ends

In a total volume of 60 µl, the recovered cleaned cDNA was mixed with 6 µl of 10 X T4 DNA polymerase buffer (700 mM Tris-HCl pH7.0, 100 mM $MgCl_2$ and 50 mM DTT), 3 µl of 2 mM dNTPs and 1 µl of T4 DNA polymerase (UBS). The reaction was incubated at 37°C for 20 min.

iii. Ligation of the adapters

To purify the cDNA , the reaction was run on 1.3% TAE agarose, the bands were cut out, 'gene cleaned' and taken up in 14 µl of milliQ water. 1 µl of this was used in a ligation reaction with 1.7 µl of the 8 mer, 3.3 µl of the 12 mer (trying to use equamolar amounts of each) with 32 units of T4 DNA ligase (New England Biolabs, 40 units/µl) in a total volume of 20 µl. The reaction was incubated for 3-4 h at RT.

iv. Ligation to vector

The adapter-ligation reaction was run on a 1.3% TAE agarose gel to separate the adapter-ligated cDNA from the non ligated adapters. The cDNA bands were cut out and 'gene cleaned' and 10 ng of the insert was ligated to 50 ng of vector in a final volume of 10 µl with 16 units of ligase (same enzyme and conditions as above). The ligation conditions were usually set up in a way that the molar ratio of insert:vector was at least 3:1. Five µl of the ligation reaction was used to transform 100 µl of frozen competent *E. coli* DH5α cells. Colonies were picked and grown, and plasmid DNA was then isolated as in a previous section.

b. Sequencing of V-region sequences-specific probes

i. Preparation of single stranded templates

pHAS has an M13 origin of replication, which means that under the right conditions (provided by *E. coli* TG2 and the helper phage) viral proteins activate the M13 origin of replication and trigger a 'simulated' phage infection cycle resulting in the production of single stranded plasmid DNA which gets packaged and is later isolated and purified. Two ml of 2xYT/MT (no antibiotics) were inoculated with 0.2 ml of an overnight culture of TG2 and grown for 1 h at 37°C on a tube roller. The culture was super-infected with helper phage R408 (0.2 ml of 5.10^{11}pfu/ml) and grown on the roller for 6-8 h at 37°C for the production and packaging of single stranded plasmid DNA . The culture was poured into Eppendorf tubes and the debris of lysed cells were pelleted twice at 4°C in a microfuge at top speed for 7 min each, the liquid being transferred to a new tube each time. The phage particles containing the pHAS DNA were then precipitated by adding 0.3 ml of 20% PEG (8000), 2.5 M sodium chloride, the tubes were mixed and incubated on ice for 15 min. The phages were pelleted twice in a microfuge at 4°C , top speed for 15 and then for 7 min, with the liquid drained before and after the second spin with the aid of a pulled out Pasteur pipette. The phage pellet was then resuspended in TE and incubated with RNase A (Boehringer) at 37°C for 30 min to destroy any bacterial RNA. Samples were then extracted twice with phenol, twice with 1:1 phenol:Cevag and twice with Cevag, ethanol precipitated in the presence of 0.3M sodium acetate (pH5.2) and rinsed with 1 ml of 70% ethanol. The final pellet was resuspended in 30-50 µl of milliQ water and 1-2 µl of this solution were run on a 0.7% agarose gel to estimate DNA concentration.

ii. Making and running of sequencing gels

Six-8% denaturing polyacrylamide gels were usually made. For a 6% gel, 15 ml of 40% acrylamide/2% N,N'-methylenebisacrylamide stock solution was mixed with 47g of urea in water with the final volume adjusted to 100 ml. After all components were dissolved, the solution was filtered through a 0.22 µm filter under vacuum. Ammonium persulfate (1 ml of 10% w/v solution in water) and 40 µl of TEMED were added, mixed

and poured into a squeeze bottle ready for the pouring of the gel. A shark's tooth comb was used with 0.4 mm spacers to cast the gel. The glass plates were taped together at the outer edges to avoid leaking and the gel was poured using the squeeze bottle. The gel was left at least 1 h to overnight to polymerize, mounted on a home-made system or on a BRL S-2 system, and run for 1-6 h. The gels were run at 60 watts constant power using an ECPS 3000/150 power supply (Pharmacia). Gels were fixed by soaking in 10% acetic acid, 10% methanol solution for 15 min and then dried at 80°C under vacuum using a slab gel drier (Hoefer Scientific Instrument, San Francisco, CA). Kodak X-ray (XAR-5) film was then exposed at RT for at least 18 h before developing.

c. Amplification of constant-region probes

Oligos were designed to amplify the first few 5' hundred bases of the mouse kappa and the gamma1 constant regions (see table 4 for oligo sequences). K5' + K3' were used with pJRC45 as their template (see previous section) to amplify the kappa, and the 'IgG1/3-5'C' + 'IgG1-3'C' primers were used with pJRC52 to amplify the gamma1 constant region. Five ng of plasmid DNA was used in the amplification reactions with 1 min at each of 94, 72 and 55°C for 25-30 cycles. However after 13 cycles, the extension time for gamma1 was changed to 2 min.

2. Screening and rescreening of B [PK3B] and PK99H cDNA libraries

Screening was performed exactly as described in the previous section except for the following changes: two nitrocellulose filters, S&S NC pore size 0.45, were used to adsorb phage from each plate. Prehybridization and hybridizations were done in a 42°C water bath, and 3 washes of 0.5-1 h were done at 65°C.

Plaques hybridizing to the probes were considered positive for possessing antibody sequences and some were picked and phage was eluted, titred, plated. Phage were adsorbed to filters from two plates for each positive plated plaque. Filters were probed and screened again for positive plaques, and well-separated, positive plaques were picked and eluted from the agar plug as in the previous section.

3. Confirming the identity of the Positive Clones

The next step was to sequence some of the positive plaques. To increase the probability of getting the complete variable sequence and to apply another level of screening before the actual sequencing, positive plaques were analyzed in two ways: 1) an amplification reaction was performed on crude phage lysates to determine which plaques contained the largest size inserts, these were "zapped out" and the resulting plasmids were mapped again with restriction digests and/or amplification reactions, 2) a Southern transfer and hybridization was performed on a restriction digest of the "zapped out" plasmids using the C-region probes.

Fifty µl of eluted phages were added to 50 µl of milliQ water, boiled for 10 min and 5 µl of this phage lysate was used in the amplification reaction. T3 and T7 were used as primers with an annealing temp of 55°C, this was repeated for 35 cycles. In vivo excision was performed on three plaques with the largest-size inserts for each of the heavy and light chain clones. Qiagin Midi preperations. or, alkaline lysis minipreparation (see previous section) were performed on all zapped plasmids. The plasmids were mapped with C-RT-PCR (with T7 and T3) and/or with restriction digests.

*Eco*RI digests of the positive plasmids were run on a gel and a double transfer to an Hybond-N membrane was performed. A membrane was placed on one side of the gel, followed by filter paper and paper towels. The pile was then flipped upside down and another membrane was laid on the other side of the gel, followed by filter paper and paper towels (same conditions and solutions used in the northern transfer). The transfer was interrupted after 72 h and the DNA was cross linked to the membrane with the use of a Stratalinker. Prehybridization, hybridization and washings were done as in previous sections except that higher than 65°C washing temperatures were used. Clones that gave positive results in both analyses were sequenced using ^{35}S-dCTP, in single (311) or double stranded sequencing.

F. Inverted-RT-PCR, cloning and sequencing of D [CD4-TT], E [Exo-s], and P [18B-13-41] V_H and V_L sequences

Double stranded cDNA was synthesized and purified by a spermine precipitation. A necessary proceeding step for the I-RT-PCR is the blunting and self-ligation of the cDNA molecules (Figure 8). Blunting and subsequent purification of the blunted cDNA molecules were done as described in previous sections.

1. Circularization of the cDNA molecules

According to Ochman (288) to get self ligation, the concentration of the cDNA in solution should be low. cDNA concentration was estimated by spotting 1 µl on an ethidium bromide plate (308). Ligation reactions were set up as in previous section except for the cDNA concentration which was set at 0.5 ng/µl, and for every 200 µl of total reaction 1 µl of T4 DNA ligase (NEB, 40U/µl) was used. The reaction was allowed to continue overnight at 15°C. Based on reference (312), for better amplification results, the cDNA was nicked by heating up the reaction to 95°C for 30 min before starting the amplification process.

2. Inverted-RT-PCR

The amplification reaction was done following the manufacturer's instructions for *Pfu* DNA polymerase (Stratagene). 2.5 ng of the self-ligated cDNA was used in a 50 µl reaction. The final concentration of dNTPs was 50 µM and 0.5 µM for each primer. 0.5 µl of *Pfu* was added last, the components were mixed and the amplification was repeated 30 times for 1 min at each of 94, 72 and 55°C (1.15 min at 72C° for heavy chain amplification). For details on the primers used, see chapter 3.

3. Cloning and sequencing of the V_H and V_L amplified sequences

The amplification reactions were run on 1.2% TAE agarose gels. Bands of the correct size were cut out and the DNA was extracted from the agarose with the Gene Clean kit. DNA was digested with either *Eco*RI/ *Sal* I for the light chain, or *Pst*I/*Xho*I for the heavy chain. The digests were run on 1% LMP TAE agarose. The

digested bands were cut out from the gel, melted and ligated (see pervious sections) to SK⁻ vectors digested with the appropriate restriction enzymes.

TG2 cells were transformed, colonies were picked and grown, and half the culture was used for Triton lysis plasmid minipreparation for a restriction-digest-mapping. The other half was reserved for ssDNA preparation and single stranded sequencing.

G. The expression of chimeric antibodies

The construction of the different expression vectors is detailed in chapters 4 and 5. Procedures used in the construction steps (e.g. restriction digests, blunting and ligation reactions, etc.) were performed as detailed in previous sections. Only modifications or additions to these techniques will be outlined below.

1- Construction of version.a vectors and the cloning of the B [PK3B] V-regions

Figures 33-39 are schematic representations of steps leading to the construction of version.a vectors and the cloning of the B V-regions; also see chapter 4. Exceptions to previously detailed techniques include: 1) depending on the restriction enzyme used, DNA sometimes had to be extracted with cevag and ethanol precipitated prior to performing some restriction digests (e.g. performing a *Kpn*I digest after a *Xho*I digest, see figure 33), 2) before some *E. coli* DH5α transformations, ligation conditions were adjusted to allow for a quick restriction digest that would reduce the cloning background by linearizing unwanted clones (e.g. in figure 33 a *Pst*I and/or a *Sst*I and a *Sal* I digest in figure 34 were performed after the first ligation step)., and 3) all amplification reactions were done using *Pfu*.

a. Dephosphorylation of vector ends

In a 200 µl reaction 0.4 units of bacterial alkaline phosphatase (USB) was used with USB supplied buffer according to the manufacturer's instructions. The reaction was incubated at 37°C for 1 h, followed by one phenol/cevag, and two cevag extractions, ethanol precipitation in the presence of 0.3 M sodium acetate and a rinse with 1 ml of 75% ethanol.

2. Construction of version.b vectors and the cloning of the V-regions

Figures 40-43 are schematic representations of steps leading to the construction of version.b vectors and cloning of the V-regions; also see chapter 4. One exceptions to previously detailed techniques will be addressed in this section: when "gene cleaning" a small piece of DNA (e.g. the *Bam*HI site containing leader in figure 40) the 'Mermaid clean' (Bio 101 inc, La Jolla) kit was used, since recovery with the Gene Clean kit was too low.

3. Construction of version.c vectors and cloning of the V-regions

Figures 44-48, are schematic representations of steps leading to the construction of version.c vectors and the cloning of the V-regions. See also chapter 4 and 5.

4. Production of recombinant proteins

a. Cell culture media and solutions

COS cells were maintained in DME medium. DME (Gibco) with 3.7 g/l of sodium bicarbonate was called plain DME, while complete DME had 10% (v/v) foetal calf serum (Hyclone, heat inactivated), L-glutamine (Gibco) 1/100 and penstrep (Gibco, a mixture of penicillin and streptomycin) 1/100.

In transfection experiments, PBS medium (Gibco, powder), Trypsin made up as 1/10 in PBS (Gibco), Trypan blue (Gibco) and Nutridoma media supplement (Nt-sp) used instead of FCS, Boehringer, 100X concentration.

b. Thawing, seeding, and splitting (subculturing) of COS cells

Frozen vials (3-4 XI0^6 cells/vial) were thawed quickly in a 37°C water bath and aliquoted into 2 medium size plates containing 25 ml of complete DME.

COS cells were monitored daily using the inverted microscope. For best results, cells were split before 100% confluency was observed, and before the media turned yellowish. COS cells are adherent cells, so in order to devide them, trypsin had to be used. DME was decanted, cells were washed twice with 10 ml of PBS. To disperse the cells 7 ml of 1/10 trypsin in PBS were added and the plates were incubated at 37°C for

10-15 min or until the cell layer started to lift off and cells were dispersed by pipetting. The cell suspension was collected, an equal volume of complete DME was added, mixed and the cells were pelleted in a Beckman GH 3.8 rotor, at 183 xg for 5 min at 4°C. The pellet was resuspended in 5 ml of complete DME. To determine cell concentration, 10 µl of the cell suspension was mixed with 10 µl of Trypan-blue, 80 µl of PBS and 10 µl of the suspension was counted with the use of a haemocytometer (blue cells are dead ones, they were not counted). Plates were also seeded by inoculating the medium with cells that were estimated to cover 10% of the plate surface area.

c. DEAE Transfections of COS cells

Plasmid DNA was prepared either by the use of QIAGEN kits (see previous sections), or by a modified large-scale alkaline preparation of plasmid DNA (313). Bacterial cells were pelleted from a 200 ml of overnight culture grown in LB. The cell pellet was resuspended in 5.3 ml of SETL buffer (15% sucrose, 25 mM Tris-HCl, 10 mM EDTA pH8.0 and 2 mg/ml lysozyme freshly added) and incubated at RT for 10 min. Cells were lysed by the addition of 10.8 ml of lysis solution (0.2 N NaOH and 1% SDS), mixing and incubating on ice for 10 min. To the lysed cells, 6.8 ml of 3M potassium acetate, pH 5.6, was added and followed by a 30 min. incubation on ice. Debris was pelleted at 14000 xg for 15 min at 4°C and DNA was precipitated from the supernatant by transferring the supernatant to a new tube containing an equal volume of isopropyl alcohol. DNA was pelleted at 10,000 Xg for 10 min. at RT and the DNA pellet was redissolved in 1.4 ml of SET buffer (10 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH8.0). The redissolved DNA was centrifuged in a microfuge for 1 min at 16,000 xg to remove debris and the supernatant was incubated at 42°C for 30 min with 10 µl of 10 mg/ml RNase A. The liquid was split into two Eppendorf tubes and extracted twice with 1:1 phenol:Cevag, and DNA was precipitated from the aqueous layer with isopropyl alcohol in the presence of 3M sodium acetate. The pellet was finally rinsed with 1 ml of 70% ethanol and air dried.

For transfecting one large (150x15mm) plate, 20 ml of DME in a 50 ml conical tube were mixed with 5-20 µg of plasmid DNA. DEAE (final concentration of 0.5 mg/ml) and chloroquine (final concentration of 100 µg/ml) were added, mixed and now the mixture was ready to be poured over COS cells rinsed with plain DME , if bottles were used, then 11 X 10^6 cells were seeded and 55 µg of DNA was used/bottle. Media was aspirated from the plates (or bottles) with 80-100% cell confluency and the cell layer was washed twice with plain DME. DME with DNA , DEAE and chloroquine were added to the plate (no chloroquine to the bottles) and incubated at 37°C for up to 4 h (7-8 h for bottles) so that the DNA would adhere to the cell layer (if incubated longer than that, the chloroquine might kill the cells). Media was aspirated and the cells were shocked with 7 ml of ice cold 1/10 DMSO in PBS. The DMSO was left on the cell layer for 2 min, after which it was aspirated and 25 ml of DME + 10% FCS were added (50 ml for bottles) and incubated at 37°C for 1.5-4 h. Media was aspirated again, cells were washed twice with DME. DME with penstrep and Nt-sp (as a supplement instead of FCS) were added to the cells (5.5-6 ml for the plates and 20 ml for the bottles). The bottles were gassed 3-5 s with CO_2 and after 72-96 h , the medium (COS-cell supernatant) was collected and cells and debris were pelleted by a 10 min centrifugation in a Beckman GH 3.8 rotor, at 183 xg at RT.

d. Concentration of recombinant proteins in COS-cell supernatant

COS cell supernatants were placed in prewashed dialysis bags (of 1000 M.Wt. cut off) and secreted proteins were concentrated against solid PEG 8000 (BDH, Cat # B800-16-34) until only 1/5 - 1/10 the original volume was left. The bags were then dialyzed against PBS (pH7.2) with 3 changes of 1-2 liters over a period of 12-24 h.

The Centriprep system (Amicon) was sometimes used, however, it took longer than the PEG.

5. Detection and analysis of recombinant proteins

To test for the secretion and the binding of the chimeric proteins, two types of enzyme-linked immunosorbent assays were carried out. Sandwich ELISA to test for the secretion, and competitive ELISA to test for binding of the chimeric proteins to *Pseudomonas aeruginosa* PAK pili. Western analysis was also carried out to check for protein size and antibody assembly.

a. Sandwich enzyme-linked immunosorbent assay (ELISA)

This assay involved four basic steps with frequent incubations and washes in between:

Coating: ELISA microtiter plates (COSTAR, flat bottom, high binding) were coated with a 100 µl/well of rabbit anti human IgG H+L (Jackson ImmunoResearch Laboratories,Inc. 309-005-082), diluted in PBS to a final concentration of 1 µg/well. Plates were incubated for 1 h at 37°C.

Blocking: The coating mixture was removed and 100 µl of 1% BSA/PBS was added. Plates were incubated at 4°C overnight.

Binding: Wells were washed twice with PBST (PBS+0.4% (v/v) Tween 20) filling the wells with the help of a squeeze bottle. A 100 µl of serial dilutions (made in 1% BSA/PBS) of sups containing the recombinant proteins were added to each well. Plates were incubated for 1h at 37°C.

Detection: Wells were washed twice with PBST. 100 µl/well of the secondary antibody (Peroxidase-conjugated-goat anti-human IgG [H+L], Jackson lab. 109035088, 1/5000 dilution) was added. Plates were incubated for 50 min to 1 h at 37°C. Three washes with PBST were followed by the addition of 100 µl of the substrate buffer (11.8 mg/ml of NaH_2PO4xH_2O, 5.88 mg/ml of citric acid, 1/10000 H_2O_2 , 1 mg/ml of ABTS powder, pH 4.2). Plates were incubated at RT for color development. If needed , the reaction was terminated by the addition of 50 µl of stop solution (42 mg/ml citric acid). Absorbance readings were taken at 405nm, using the Titertek Multisystem Plus MK2, from Flow laboratories.

b. Competitive ELISA

Competitive ELISA was performed according to the modifications introduced by Doig *et al* . (105) to the original method introduced by Voller *et al.* (314).

Coating: Wells were coated with 100 µl of 1/2000 dilution (0.2 µg/well) of goat anti-human IgG Fc (Cappel, 55071 06010121), or with 1/4000 of anti-mouse Ig (polyvalent, rabbit anti-mouse Ig antiserum Cedarlane Lab. Cat # CL 6002) in a 0.01 M carbonate buffer, pH 9.5. Plates were incubated at 37°C for 1-2 h or left overnight at 4°C.

Blocking: Wells were washed three times with buffer A (0.05% [w/v] BSA in PBS, pH 7.4) and blocked with 100 µl of 3% BSA in PBS (w/v) at 4°C overnight.

Coating: Wells were washed three times with buffer A, all washings were done with the Mini-Wash Skater (Norway). 100 µl serial dilutions (made in buffer A) of sups containing the recombinant proteins, or serial dilutions of mouse antibodies were added to each well. Plates were incubated at 37°C for 1-1.5 h.

Blocking: Wells were washed two times with buffer A and blocked again with 100 µl of 0.5 % dry skim milk / PBS at 37°C for 1 h.

Binding: Wells were washed three times with buffer A. 100 µl of 1/1000 dilution of biotinilated PAK pili (1.76 mg/ml stock concentration) were competed with varying concentrations of non-biotinilated PAK pili (50 µl of biotinilated PAK pili were premixed with 50 µl of one of the dilutions of the non biotinilated pili). Plates were incubated at 37°C for 1 h.

Detection: Wells were washed three times with buffer A. Alkaline phosphatase-streptavidin conjugate (Gibco BRL) was diluted 1/3000 in buffer A and 100 µl of that was added to each well. Plates were incubated for 1 h at RT. Wells were washed three times with buffer A , followed by the addition of 100 µl of substrate (1 mg/ml of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.6). Plates were left on a shaker at

RT for color development. Absorbance readings were taken at 405nm, using Titertek Multisystem Plus MK2, from Flow laboratories.

c. Western blots of chimeric proteins

Polyacrylamide separating and stacking gels were prepared following standard protocol (303). Sups from COS cells were mixed with load dye (for 100 ml of 2X load dye, 25 ml of 4X Tris.Cl/SDS, pH6.8 were mixed with 20 ml of glycerol, 4 g of SDS and 1 mg of Bromophenol Blue) and loaded to be run under non-reducing conditions. To run the samples under reducing conditions, 1.5 µl of β-mercaptoethanol was added to every 10 µl of sample (including load dye), mixed and heated to 85°C for 15 min before loading. Bio-Rad's prestained and regular unstained SDS-PAGE protein markers were usually run alongside the samples. Gels were run at 40 mA until the smallest molecular weight band was run off the gel.

The gel was washed in 1 X transfer buffer (303) at RT for 15 min on a shaker. 3 MM filter papers were cut to the size of the gel and soaked in 1 X transfer buffer. Nitrocellulose membrane (PROTRAN, Mandel scientific BA 85, pore size 0.45 µm) was cut to the size of the gel and wetted in water then in 1 X transfer buffer. To prepare the gel for the electrotransfer system (Bio Rad), the white pads of the transfer system were also wetted in 1X transfer buffer, and one was laid down on the black side of the cassette followed by 2-3 filter papers, the gel, the membrane, 2-3 filter papers and the second white pad. If another gel was also being transferred, then after the second 2-3 filter papers, the other gel, membrane, 2-3 filter papers and then the white pad would be placed. The ice pocket was filled with ice, the chamber was filled with 1 X transfer buffer and the system was run at 200 mA for 40 min.

Visualizing the protein markers: After the transfer, the membrane was soaked with the protein side up in Ponceau dye (0.5 g of Ponceaus in 1 ml of glacial acetic acid) for 1 min. Excess dye was washed off with water and the bands of the protein markers were marked on the membrane with a filter pen.

Blocking: The membrane was placed in a 50 ml conical tube (with the protein side facing inward) and 10 ml of PBST + 10% dry skimmed milk were added. The tube was placed on the tube roller overnight at 4°C.

Binding: The membrane was washed twice with PBST and 1 ml of PBST containing 1 µl of peroxidase-conjugated-goat anti-human IgG [H+L], Jackson lab. 109035088, 1/5000 dilution was added. The membrane was incubated for 1 h on the tube roller at 37°C.

Detection: The membrane was given a 15 min wash with PBST, followed by four more washes of 5 min each at RT. The membrane was then transferred to a new tube and the ECL western blotting detection reagents 1 and 2 (Amersham) were used according to the manufacturer's instructions for developing. The membrane was warped in Saran Wrap and different exposures were taken using ECL film.

CHAPTER III

CLONING AND SEQUENCING OF THE HEAVY AND LIGHT-CHAIN VARIABLE GENES

A. *Introduction*

In this thesis we studied five different mouse monoclonal antibodies, all with specificity for *Pseudomonas aeruginosa* pili. The antibodies studied include PK99H, PK3B (abbreviated B), CD4-TT (abbreviated D), Exo-s (abbreviated E), and 18B-13-41 (abbreviated P, also known as Pertussis). This chapter describes the approaches used to obtain heavy and light chain variable-region (V_H and V_L) cDNA clones for each of these antibodies, as well as the cDNA sequences themselves. As described in more detail below, a traditional cloning approach (the construction and screening of cDNA libraries) was used to clone cDNAs for PK99H and B, whereas a more modern PCR approach (inverted polymerase chain reaction) was used for D, E and P. In all cases except PK99H we were able to obtain the appropriate DNA sequences to proceed with construction of chimeric antibodies.

B. *Cloning of heavy and light-chain variable region cDNAs of the B [PK3B] and PK99H antibodies*

B and PK99H V-region sequences were isolated by constructing and screening cDNA libraries. Two types of probes were used to screen these libraries, constant region probes and V-region sequence-specific probes. To check for the quality and the availability of the antibody messages in the RNA preparations, northern blots were first prepared and probed with constant region probes. For the B antibody, bands were obtained using both heavy chain constant region probe ($C\gamma1$) as well as kappa constant region ($C\kappa$) probe (data not shown). In the case of PK99H, a band was obtained upon probing with the $C\kappa$ probe, however, no clear band, only a faint smear, was seen when the $C\gamma1$ probe was used (data not shown). This experiment confirmed that the probes

were functional, and would be able to detect cDNAs for the H and L chains when used to screen libraries. It also confirmed that the cell lines were generating heavy and light chain transcripts (at least for the B hybridoma).

The motivation to use V-region probes as well as constant region probes was two fold: 1) probes from the 5' region of a mRNA were more likely to detect full length cDNA clones. 2) V-region sequence-specific probes were more likely to detect expressed chains rather than aberrant chains transcribed from the myeloma fusion partner (see later sections on PK99H and P antibodies light chains). In collaboration with Dr. Randy Irvin's laboratory, B and PK99H antibodies were purified and subjected to N-terminal sequencing. Although the B heavy chain was blocked, protein sequences were obtained for the other three chains, and this is shown in figure 13. Based on this data a reverse translation was performed, and a series of V-region specific degenerate oligonucleotides were designed, which are also shown in figure 13. The various degenerate oligos in combination with appropriate constant region oligos (K5'constant, IgG1 5' constant, Table 4) were used in a RT-PCR containing first strand cDNA from each of B and PK99H RNA preparations.

The RT-PCR reaction containing the B light chain and Cκ primers generated a fragment of about 350 base pair fragment which was cloned (designated clone 126) and sequenced from the 5' end. Partial DNA sequence for clone 126 is shown in figure 14. The deduced protein sequence of clone 126 indicated that it encoded a protein essentially identical to the original sequenced B kappa chain.

The RT-PCR reaction containing the PK99H heavy chain and Cγ1 primers also generated a DNA fragment of about 350 base pairs which was cloned (designated 8a5) and sequenced from the 5' end. A partial DNA sequence for clone 8a5 is shown in figure 15. Again the deduced protein sequence of the cloned PCR fragment indicated that it encoded a protein essentially identical to the original sequenced PK99H heavy chain. In contrast, 3 different combinations of PK99H V-region sequence-specific and

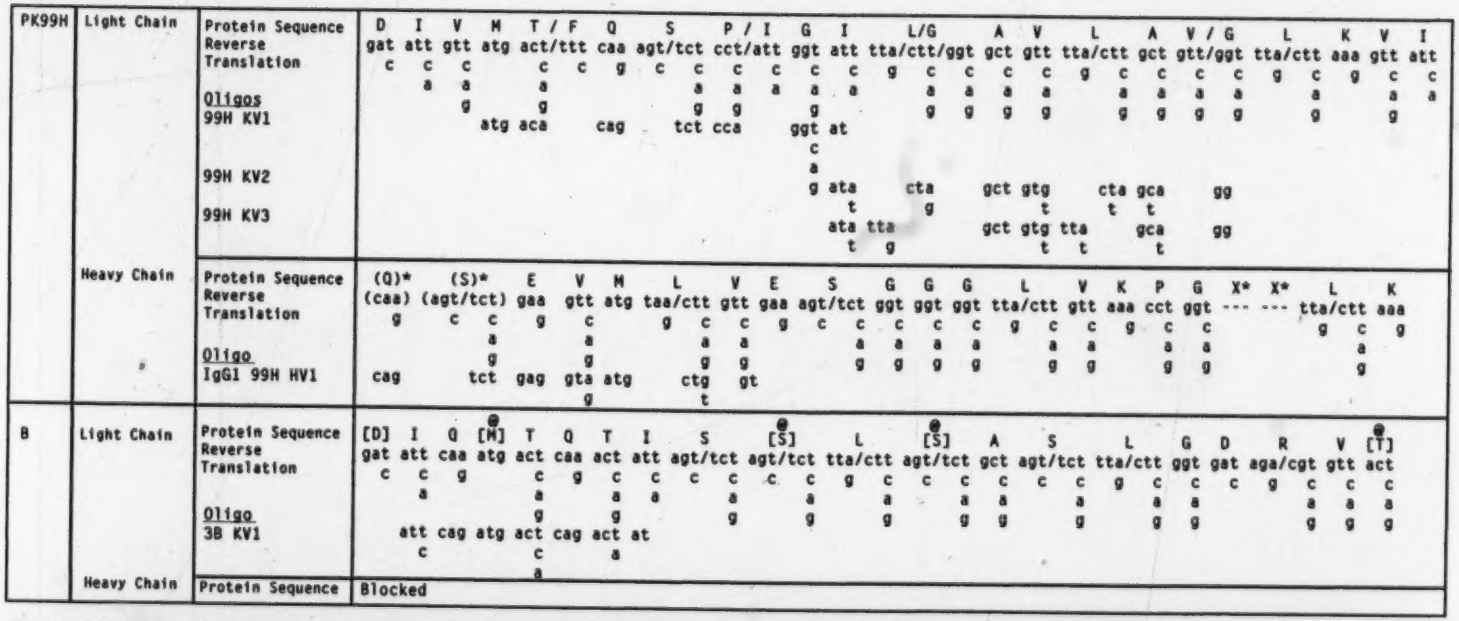

PK99H
Light Chain
Protein Sequence
Reverse Translation
Oligos
99H KV1
99H KV2
99H KV3
D I V M T / F Q S P / I G I L/G A V L A V / G L K V I
gat att gtt atg act/ttt caa agt/tct cct/att ggt att tta/ctt/ggt gct gtt tta/ctt gct gtt/ggt tta/ctt aaa gtt att
atg aca cag tct cca ggt at
ata cta gct gtg cta gca gg
t g t t t t
ata tta gct gtg tta gca gg
t g t t t
Heavy Chain
Protein Sequence
Reverse Translation
Oligo
IgG1 99H HV1
(Q)* (S)* E V M L V E S G G G L V K P G X* X* L K
(caa) (agt/tct) gaa gtt atg taa/ctt gtt gaa agt/tct ggt ggt ggt tta/ctt gtt aaa cct ggt --- --- tta/ctt aaa
cag tct gag gta atg ctg gt
g t
B
Light Chain
Protein Sequence
Reverse Translation
Oligo
3B KV1
[D] I Q [M] T Q T I S [S] L [S] A S L G D R V [T]
gat att caa atg act caa act att agt/tct agt/tct tta/ctt agt/tct gct agt/tct tta/ctt ggt gat aga/cgt gtt act
att cag atg act cag act at
c c a
Heavy Chain
Protein Sequence
Blocked

Figure 13. N-terminal amino acids of the sequenced proteins in antibodies B and PK99H, and degenerate oligonucleotides designed to amplify V-region sequence-specific probes. The protein line represents the amino acid sequence of the N-terminus of the heavy and light chains with A/B representing ambiguous sequence assignments. * For PK99H heavy chain, we arbitrarily assumed the first two amino acids (shown in brackets) which represent the (-2), (-1) residues that most commonly occur in immunoglobulin sequences. @ For B, the amino acids indicated in square brackets were not clear in the protein sequence, but represent the most likely candidates. X* represent undetermined amino acids.

Protein Sequencin	[D] I Q [M] T Q T I S [S] L [S] A S L G D R V T
Clone 126	I Q M T Q T I S S L S A S L G D R V T I T C R A S Q D V K N ggg atc cag atg act cag aca ata tcc tcc ctg tct gcc tct ctg gga gac aga gtc acc atc act tgc agg gca agt cag gac gtt aaa aat
PCR oligo	att cag atg act cag act at c c a a
cDNA library clone	gat atc cag atg aca cag act ata tcc tcc ctg tct gcc tct ctg gga gac aga gtc acc atc act tgc agg gca agt cag gac gtt aaa aat D I Q M T Q T I S S L S A S L G D R V T I T C R A S Q D V K N

Figure 14. Partial DNA and deduced amino acid sequence of the PCR amplified 126 compared with the original protein sequence and with the DNA sequence of B light chain clone subsequently obtained from our B library. Square brackets indicate questionable amino acids obtained from protein sequencing. For the complete sequence of the cDNA library clone see Figure 17. The bold letters signify those bases which are different between the library clone and the amplified 126 clone.

Protein Sequence	E V M L V E S G G G L V K P G X X L K
Clone 8a5	E V M L V E S G G G L V K P G G S L K L S C A A S G F T F S gag gtg atg ctt gtg gag tct ggg gga ggc ttg gtg aag cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc agt
PCR oligo	gag gta atg ctg gt g t
cDNA library clone #2	gaa gtg atg ctg gtg gag tct ggg gga ggc tta gtg aag cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc agt E V M L V E S G G G L V K P G G S L K L S C A A S G F T F S

Figure 15. Partial DNA and deduced amino acid sequence of clone 8a5, compared with the original protein sequence and with the cDNA sequence of a PK99H heavy chain clone subsequently obtained from our PK99H cDNA library. For the complete sequence of the cDNA clone #2 see Figure 19. The bold letters signify bases which are different between the library clone and the amplified 8a5 clone. For a comparison of the complete sequence of 8a5 relative to that of clone #2, see Figure 21. X is undetermined amino acid, obtained from protein sequencing.

Cκ primers were used in RT-PCR reactions, however, we were unable to generate any PCR fragment representing the PK99H light chain gene. Nevertheless, we were able to amplify γ actin message from the PK99H RNA, indicating that the RT-PCR reaction as well as the mRNA were intact.

As a negative control, we performed RT-PCR reactions on mRNA from the myeloma fusion partner NS1, using all of the variable and constant region primer pairs outlined above. In this case, only the γ actin primers yielded a PCR product.

To obtain the necessary clones, cDNA libraries were constructed in λZapII*Sfi* IB, starting with total cellular RNA (in the case of PK99H) or poly A+ RNA (in the case of B). Table 5 summarizes the essential data for these libraries, as well as the results obtained when they were screened with either V-region or constant region probes. Depending on how many positive plaques were obtained in the initial screening, either all or a subset of these were picked, re-plated, and rescreened to obtain confirmed positive clones as isolated plaques.

Plaques that were positive upon rescreening were analyzed by PCR (using T3 and T7 primers) to determine the cDNA insert size in each case. For each group of clones, the three individual plaques with the largest sized inserts were *in vivo* subcloned (e.g "zapped out") into pBluescriptSK$^-$ and insert sizes were mapped by restriction analysis. These insert sizes are also summarized in Table 5. The same restriction digests were also separated by agarose gel electrophoresis, transferred to nylon membranes, and the resulting clone blots probed with the Cγ1 or Cκ probes as appropriate.

For the B heavy chain clones, originally obtained by hybridizing to the Cγ1 probe, all tested inserts hybridized to the γ1 probe, and all were 1.9 Kbp long. Likewise, all three light chain clones (originally obtained by hybridizing to the V-region sequence-specific probe 126) had inserts that were positive with the Cκ probe, and all were 1.6 Kbp long. For the PK99H heavy chain clones (originally selected with the V-region sequence-specific probe 8a5), only one of the original three clones (clone #1) contained

Table 5. cDNA library screening results for B and PK99H

cDNA library	B (PolyA+ RNA)		PK99H (Total RNA)		
Period over which cDNA library was constructed and screened	June 92-October 92		June 92-October 92		
Library size	4×10^5 independent clones		6.6×10^4 independent clones		
Probe	V_L-region specific probe (126)	Gamma 1 ($C\gamma 1$)	V_H- region specific Probe (8a5)	Gamma 1 ($C\gamma 1$)	Kappa ($C\kappa$)
Positive plaques upon initial screening	~300/150,000	~200/150,000	4/66,000	1/66,000	>500/66,000
Plaques picked for re-screening with the same above probe	10	20	4	1	20
Positive plaques upon re-screening	10/10	20/20	3/4	0/1	19/20
Insert sizes of light-chain clones zapped out	#6- 1.6 Kbp #7- 1.6 Kbp #9- 1.6 Kbp		#7- 1.4 Kbp #9- 1.4 Kbp #11- 1.4 Kbp		
Clones with inserts which cross hybridize with $C\kappa$ probe	#6, #7, #9		#7, #9, #11 @		
Insert sizes of heavy-chain clones zapped out	#2- 1.9 Kbp #13- 1.9 Kbp #15- 1.9 Kbp		#1- 1.5 Kbp #2- 1.8 Kbp #3- 1.9Kbp		
Clones with inserts which cross hybridize with $C\gamma 1$ probe	#2, #15 (#13 *)		#1		

* Clone #13 was not included in the Southern; but it's insert clearly would cross hybridize with the $C\gamma 1$ probe since this is how the clone was selected in the first place. @ Expected results since clones were chosen on the basis of K reactivity.

an insert that cross-hybridizes with the Cγ1 probe, and unfortunately it was relatively short (1.5 Kbp). The other two clones (clones #2 and #3) had larger insert sizes of 1.8 Kbp and 1.9 Kbp respectively. In contrast, all three of the PK99H light chain clones (originally selected with the Cκ probe) had inserts that were 1.4 Kbp long and all three hybridized to the Cκ probe.

C. *Sequences of heavy and light-chain variable region cDNAs of B [PK3B] and PK99H*

B heavy chain- A composite cDNA sequence for this V region is shown in figure 16. Of the three clones sequenced only one (#2) extended sufficiently 5' to contain the +1 codon of the VH protein. The *Sfi* IB linker was detected followed by codon +10 for clone #15 and codon +25 for clone #13. The sequence from codon +1 to +9 is thus solely from clone #2, whereas the sequence for codons +10 to +24 is the same in clones #2 and #15, and from +25 onwards the sequence is derived from all three clones. In this latter region all three sequences were identical except for one base in codon +107, where in clones #13 and #15 the triplet was TGG, but in clone #2 it was CGG.

B light chain- A composite cDNA sequence for this V region is shown in Figure 17. Clone #9 gave the complete variable sequence with the leader peptide, whereas clone #7 began within the leader. Clone #6 gave the identical sequence as the other two from codon +58 onwards, but the sequence of the most 5' region was not obtained. All three sequences were identical in the parts where they overlapped.

PK99H heavy chain- Unfortunately none of the three cDNA clones isolated represented a mature mRNA transcript, and no two of the sequences were alike. The sequences are shown in figures 18, 19 and 20. The 5' end of clone #1 (shown in figure 18) encodes a variable region sequence which does not correspond to the known protein sequence of the PK99H VH chain. At the 3' end, clone #1 contains DNA sequences

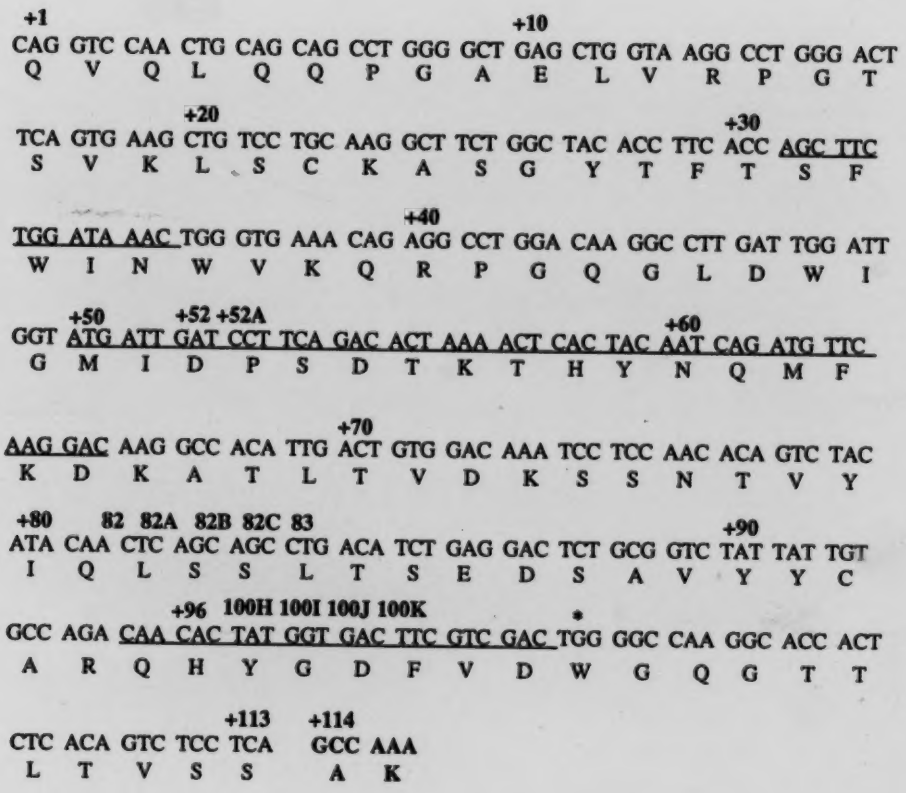

+1
+10
CAG GTC CAA CTG CAG CAG CCT GGG GCT GAG CTG GTA AGG CCT GGG ACT
Q V Q L Q Q P G A E L V R P G T
+20
+30
TCA GTG AAG CTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACC AGC TTC
S V K L S C K A S G Y T F T S F
+40
TGG ATA AAC TGG GTG AAA CAG AGG CCT GGA CAA GGC CTT GAT TGG ATT
W I N W V K Q R P G Q G L D W I
+50
+52 +52A
+60
GGT ATG ATT GAT CCT TCA GAC ACT AAA ACT CAC TAC AAT CAG ATG TTC
G M I D P S D T K T H Y N Q M F
+70
AAG GAC AAG GCC ACA TTG ACT GTG GAC AAA TCC TCC AAC ACA GTC TAC
K D K A T L T V D K S S N T V Y
+80
82 82A 82B 82C 83
+90
ATA CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAT TGT
I Q L S S L T S E D S A V Y Y C
+96 100H 100I 100J 100K
*
GCC AGA CAA CAC TAT GGT GAC TTC GTC GAC TGG GGC CAA GGC ACC ACT
A R Q H Y G D F V D W G Q G T T
+113
+114
CTC ACA GTC TCC TCA GCC AAA
L T V S S A K

FIGURE 16. DNA and amino acid sequence of B heavy chain variable region. The underlined nucleotides encode the CDRs, with CDR 1 from 31-35, CDR 2 from 50-65 and CDR 3 from 95-102. Codon 103 (with the*) is a TGG in clone # 13 and 15, but a CGG in clone # 2 which changes the amino acid from W to R, but W is the usual amino acid at this position. The location of the CDRs and the numbering of the residues were determined by consulting Seqhunt II (327), How to Identify CDRs (328) and also by comparison to other published sequences. Gamma 1 constant region begins with an alanine at position +114.

-20 -10
ATG ATG TCC TCT GCT CAG TTC CTT GGT CTC CTG TTG CTC TGT TTT CAA
M M S S A Q F L G L L L L C F Q

-1
GGT ACC AGA TGT
G T R C

+1 +10
GAT ATC CAG ATG ACA CAG ACT ATA TCC TCC CTG TCT GCC TCT CTG GGA
D I Q M T Q T I S S L S A S L G

+20 +30
GAC AGA GTC ACC ATC ACT TGC AGG GCA AGT CAG GAC GTT AAA AAT TAT
D R V T I T C R A S Q D V K N Y

+40
GTA AAG TGG TAT CAG CAG AAA CCA GAT GGA ACT ATT AAA CTC CTG ATC
V K W Y Q Q K P D G T I K L L I

+50 +60
CAC TAC ACA TCA AGA TTA CAC TCA GGA GTC CCA TCA AGG TTC AGT GGC
H Y T S R L H S G V P S R F S G

+70 +80
AGT GGG TCT GGA ACA GAT TAT TCT CTC ACC ATT AGT AAC CTG GAA CAA
S G S G T D Y S L T I S N L E Q

+90
GAA GAT ATT GCC ACT TAC TTT TGT CAA CAG GGG CAT ATA CTT CCG TGG
E D I A T Y F C Q Q G H I L P W

+100 +107 +108
ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAG CGG GCT
T F G G G T K L E I K R A

FIGURE 17. DNA and amino acid sequence of B light chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 24-34, CDR 2 from 50-56 and CDR 3 from 89-97. CDRs were assigned as in figure 16. The kappa constant region begins with an arginine at position +108. Amino acids from position -20 until -1 represent the leader peptide.

A.

GAT GTT CAG CTG GTG GAG TCT GGG GGA GGC TTA GTT CAG CCA
D V Q L V E S G G G L V Q P

GGA GGT TCC GG
G G S

B.

ATC TTG ACA AAC AGA GAC AAA TTT GAG TAT CAC CAG CCA AAA GTC ATA CCC AAA

AAC AGC CTG GCA TGA CCT CAC ACC AGA CTC AAA CTT ACC CTA CCT TTA TCC

TGGTGGCTTCTCATCTCCAGACCCCAGTAACACATAGCTTTCTCTCCACAG

FIGURE 18. DNA and amino acid sequence of PK99H V_H clone #1, obtained by screening the cDNA library. **A.** is the 5' sequence of a variable gene , which does not match the V_H protein sequence. **B.** is the 3' end of the clone, which contains an intronic sequence that is present between the γ1 exons, just 5' of the hinge region exons.

identical to intron sequences found 5' of the γ1 hinge region exons. Presence of the CH1 exon of Cγ1 apparently accounts for cross-hybridization with the Cγ1 probe.

Clone #2 (figure 19) encodes a V-region sequence which matches precisely the N-terminal amino acid sequence determined for the secreted PK99H VH protein (Figures 19 and 15). However the open reading frame extends only to codon +94, and this is followed by the heptamer and nanomer sequences of an unrearranged VH gene segment (Figure 19). The partial DNA sequence for clone #3 is shown in Figure 20. The 5' end encodes a leader sequence that is different than that of clone #2, with the L-V intron remaining unspliced. The 3' end is identical to an intron sequence that is usually located in the J region, 3' of JH4. Since clones #1 and #3 were not useful, they were not further characterized.

Further characterization of clone 8a5- Because of our failure to obtain any cDNA clones for the PK99H heavy chain, we went back and further characterized the V-region sequence-specific clone 8a5 which we had generated by RT-PCR, since it potentially contained the entire V-region sequence. The entire sequence of 8a5 is shown in figure 21, where it is compared to the sequence of PK99H VH clone #2. Although the 8a5 sequence is virtually identical to clone #2 in the 5' region, it becomes completely unlike it in the 3' portion, and it does not apparently encode a complete VH region.

PK99H light chain- Three clones were partially sequenced and were identical in the parts that overlapped. Only one clone (#7) was fully sequenced, and this is shown in figure 22. When this sequence was used to scan the GenBank database, it was found to be identical to an aberrant light chain transcript which is produced by the NS1 myeloma fusion partner. None of the three clones were pursued any further.

D. *Cloning of heavy and light-chain variable region cDNAs for the D [CD4-TT], E [Exo-s], and P [18B-13-41] antibodies*

Initially we chose to screen cDNA libraries (as described above for the B and PK99H antibodies), rather than using a RT-PCR approach, because we wished to obtain

-19 -10
ATG AAC TTC GGG CTC AGC TTG ATT TTC CTT GTC CTT GTT TTA AAA
M N F G L S L I F L V L V L K

-1
GGT GTC CAG TGT
G V Q C

+1 +10
GAA GTG ATG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG
E V M L V E S G G G L V K P G G

+20 +30
TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT
S L K L S C A A S G F T F S S Y

+40
GCC ATG TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC
A M S W V R Q T P E K R L E W V

+50 52 52A 53 +60
GCA ACC ATT AGT AGT GGT GGT AGT TAC ACC TAC AAT CCA GAC AGT GTG
A T I S S G G S Y T Y N P D S V

+70
AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC
K G R F T I S R D N A K N T L Y

+80 82 82A 82B 82C 83 +90
CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC ATG TAT TAC TGT
L Q M S S L R S E D T A M Y Y C

GCA AGA CA CACAATG AGGAAATGTTACTGTGAGCTCAA ACTAAAACC
A R

FIGURE 19. DNA and amino acid sequence of PK99H heavy chain variable region clone #2. The underlined nucleotides encode the CDRs, with CDR 1 from 31-35, and CDR 2 from 50-65 ; instead of CDR 3 we obtained DNA sequences for the heptamer and the nanomer recombination signals (shadowed bases). The first 19 amino acids (-19 to -1) are those representing the leader peptide.

A.

ATG GAC TCC AGG TTC AAT TTA GTT TTC CTT TTC CTT ATT TTA AAA
M D S R F N L V F L F L I L K

GGT AAT TTG TAG AGA TGA GTT TCT

B.

AAT ACT GTG ACT TTA AAA TGT GAG AGG GTT TTC AAG TAC TCA TTT

TTT TAA ATG TCC AAA ATT TTT GTC AAT CAA TTT GAG GTC TTG TTT

GTG TAG AAC TGA CAT TAC TTA AAG CTT AAC CGA GGA ATG GGA GTG

AGG CTC TCT CAT ACC CTA TTC AGA ACT GAC TTT TAA

FIGURE 20. DNA and amino acid sequence of PK99H V_H clone #3, obtained by screening the cDNA library. A. is the sequence of a leader peptide, with the underlined letters representing the unspliced intron. B. An intronic sequence in the J region down stream of JH4. The C is in our sequence instead of a T.

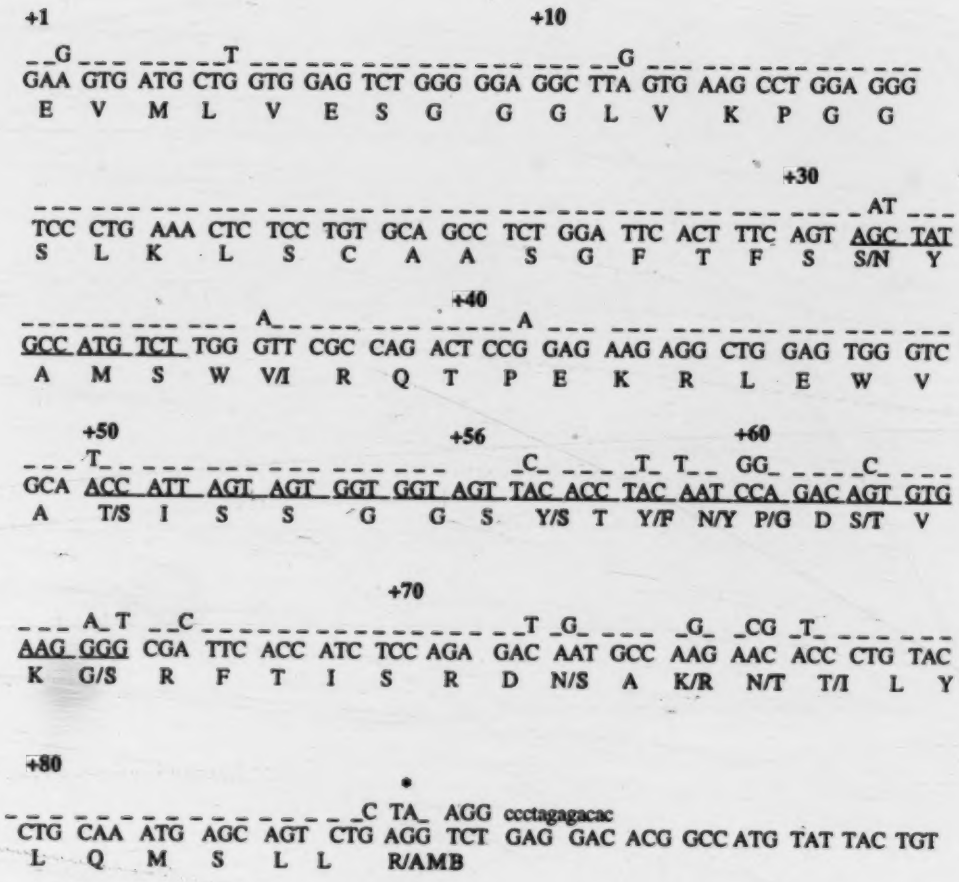

+1
+10
GAA GTG ATG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG
E V M L V E S G G G L V K P G G
+30
TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT
S L K L S C A A S G F T F S S/N Y
+40
GCC ATG TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC
A M S W V/I R Q T P E K R L E W V
+50
+56
+60
GCA ACC ATT AGT AGT GGT GGT AGT TAC ACC TAC AAT CCA GAC AGT GTG
A T/S I S S G G S Y/S T Y/F N/Y P/G D S/T V
+70
AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC
K G/S R F T I S R D N/S A K/R N/T T/I L Y
+80
CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC ATG TAT TAC TGT
L Q M S S L L R/AMB
ccctagagacac

FIGURE 21. A comparison of the DNA and amino acid sequence of the PCR amplified fragment 8a5 in comparison with PK99H V_H clone #2 obtained by screening the cDNA library with 8a5. Dashes represent identical bases, while differences are presented over the clone #2 sequence. Gap at +56, there is no 8a5 sequence for this codon. The letters in outline font are the corresponding amino acids for fragment 8a5. This 8a5 fragment was used as a probe to screen the cDNA library and pull out clone #2. * is a stop codon in the 8a5 fragment and the lower case text is the Has-LINK 12 adapter that was used in the cloning process (see Chapter 2).

```
                                                -10
ACA  CTC  CTG  TTA  TGG  GTA  CTG  CTG  CTC  TGG  GTT  CCA  GGT  TCC
 T    L    L    L    W    V    L    L    L    W    V    P    G    S

      -1
ACT  GGT
 T    G

+1                                                  +10
GAC ATT GTG CTG ACA CAG TCT CCT GCT TCC TTA GCT GTA TCT CTG GGG
 D   I   V   L   T   Q   S   P   A   S   L   A   V   S   L   G

              +20           &
CAG AGG GCC ACC ATC TCA TAC AGG GCC AGC AAA AGT GTC AGT ACA TCT
 Q   R   A   T   I   S   Y   R   A   S   K   S   V   S   T   S

    +30                                             +40
GGC TAT AGT TAT ATG CAC TGG AAC CAA CAG AAA CCA GGA CAG CCA CCC
 G   Y   S   Y   M   H   W   N   Q   Q   K   P   G   Q   P   P

                    +50                                     +60
AGA CTC CTC ATC TAT CTT GTA TCC AAC CT* GAA TCT GGG GTC CCT GCC
 R   L   L   I   Y   L   V   S   N   L   E   S   G   V   P   A

                                                +70
AGG TTC *GT GGC AGT gGG TCT GGG ACA GAC TTC ACC CT* AAC ATC CAT
 R   F   X   G   S   G   S   G   T   D   F   T   L   N   I   H

         +80                                            +90
CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT CAG CA* *TT AGG
 P   V   E   E   E   D   A   A   T   Y   Y   C   Q  H/Q  X   R


GAG *TT ACA CGT TCG *AG GGG GGA CCA AGC TGG  A*A
 E   X   T   R   S   X   G   G   P   S   W   X
```

FIGURE 22. DNA and amino acid sequence of clone #7, a V_L obtained by screening the PK99H cDNA library. The underlined nucleotides are the CDRs, with CDR 1 from 24-34, CDR 2 from 50-56 and JK2 from 94-. The first two lines with the sequence in italic are those of the leader sequence. This sequence is identical to an aberrant light chain present in the NS-1 myeloma fusion partner (317). It is identified among other things by the presence of a Y amino acid at position 23 (&) instead of the usual cysteine. * represents bases which could not be read clearly from the sequencing gel. For the full published sequence of the aberrant V_L, see figure 31.

the complete V-region sequences without changes imposed by consensus V-region oligonucleotides. We were also concerned that Taq-DNA polymerase would introduce significant numbers of mutations beyond those due to reverse transcriptase copying errors. However, this traditional approach proved to be slow and very labor intensive. As the number of antibodies we wished to put through the engineering process increased, and a new high fidelity thermostable DNA-polymerase (*Pfu*) became available, the decision was made to switch to an inverted-RT-PCR (I-RT-PCR) strategy (described in more detail in Chapter 1, also see figure 8). We anticipated that this approach would be much faster, and would not impose base-changes due to PCR primers.

At the time this work was carried out there were no reports describing the use of I-RT-PCR to clone immunoglobulin genes [since then a few have been published (315,316)], and therefore no concrete examples of effective primers which could be utilized. Initially we tested three different sets of kappa primers in a number of possible combinations. The first two sets did not have restriction sites on their 5' ends (KC5'(IPCR), KC3'(IPCR)a and KC5'(IPCR)a, KC3'(IPCR)a2, Table 3), the third set did (shown in figure 23). The rationale for this approach was that the primers containing the restriction sites would be more likely to prime in an aberrant fashion, since they were much longer (36-37 bases versus 20-25 bases for oligos without restriction sites) and had many bases which were not homologous to the target sequence. On the other hand, if the primers with the restriction sites could be used in the first instance, they would allow direct cloning from a single PCR reaction, and multiple rounds of PCR would not be required. The different primer combinations were used in I-RT-PCR reactions containing B total cellular RNA. All tested combinations worked with varying degrees, and based on these results a subset of these primers were used in I-RT-PCR reactions containing D, E and P total cellular RNA. PCR fragments of the expected size were obtained in all cases (data not shown), however, for sake of convenience, and because

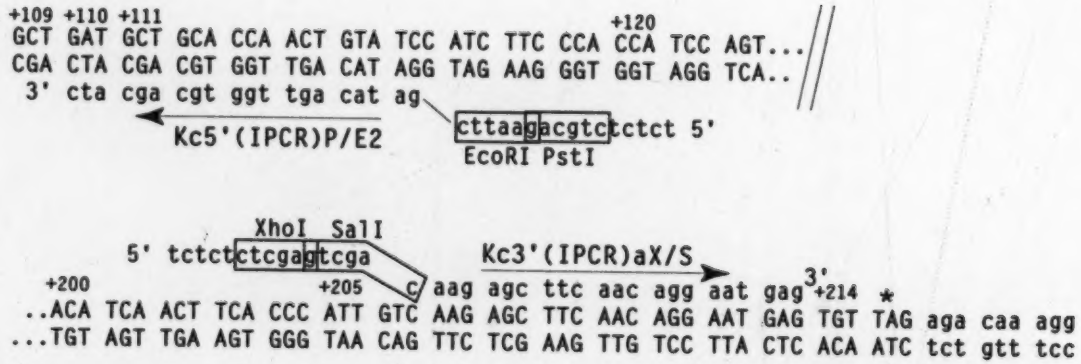

+109 +110 +111
GCT GAT GCT GCA CCA ACT GTA TCC ATC TTC CCA CCA TCC AGT...
+120
CGA CTA CGA CGT GGT TGA CAT AGG TAG AAG GGT GGT AGG TCA..
3' cta cga cgt ggt tga cat ag
Kc5'(IPCR)P/E2
cttaagacgtctctct 5'
EcoRI PstI
XhoI SalI
5' tctctctcgagtcga
Kc3'(IPCR)aX/S
3'
+200
+205
c aag agc ttc aac agg aat gag +214 *
..ACA TCA ACT TCA CCC ATT GTC AAG AGC TTC AAC AGG AAT GAG TGT TAG aga caa agg
...TGT AGT TGA AGT GGG TAA CAG TTC TCG AAG TTG TCC TTA CTC ACA ATC tct gtt tcc

Figure 23. The relative location of the I-RT-PCR primers in relation to mouse Kappa constant region sequence. The lower case letters in the sequence represent the 3' untranslated region.

they gave good yields of full-length product, fragments generated using the primer pairs containing restriction sites were digested with *Eco*RI and *Sal*I, and force cloned into pBluescript SK$^-$. At least three individual clones for each light chain were grown up and sequenced (see below).

A similar approach was used to clone the D, E, and P heavy chain V regions, which had all been shown to be of the IgM class by serological methods (Randy Irvin, personal communication). The primers shown in figure 24 were used in various combinations, and all I-RT-PCR reactions generated DNA fragments of the expected size (figure 25). For the same above mentioned reasons, fragments generated using the primer pairs containing restriction sites were digested with *Xho*I and *Pst*I and cloned and sequenced as for the light chains above.

E. *Sequences of heavy and light-chain variable region cDNAs for the [CD4-TT], E [Exo-s], and P [18B-13-41] antibodies*

D heavy-chain- See figure 26 for the composite V_H sequence. Three clones gave exactly the same sequence, except that at codon +112, clones #1 and #4 had the TCT triplet, while in clone #5 the triplet was TCC which creates no change in the corresponding amino acid.

D light-chain- This DNA sequence is shown in figure 27. Three clones were sequenced, and all sequences were identical.

E heavy-chain- See figure 28 for the complete sequence. Three clones were sequenced, and all sequences were identical.

E light-chain- Three clones were fully sequenced; clones #5, #6, and #7. The sequence of clone #5 is shown in figure 29, beginning at the +1 codon. The sequences of clones #6 and #7 are essentially identical to that of #5 from the +6 codon inclusive to the beginning of the constant region. From codons +1 to +5, these clones had the sequence (TGC TAA TAT TTG CA)C, with the sequence in brackets representing the kappa 3' untranslated region. One other difference between the three sequence occurred

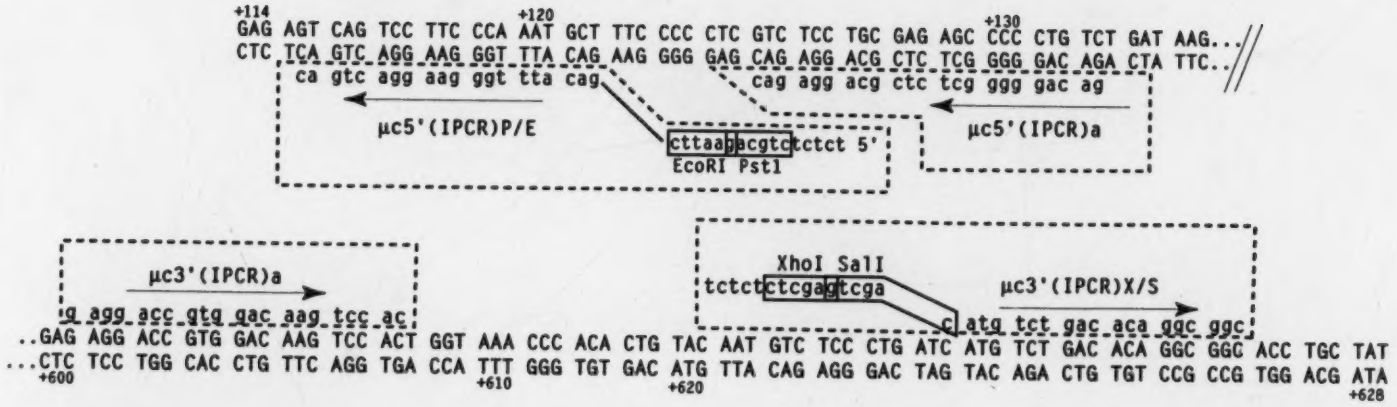
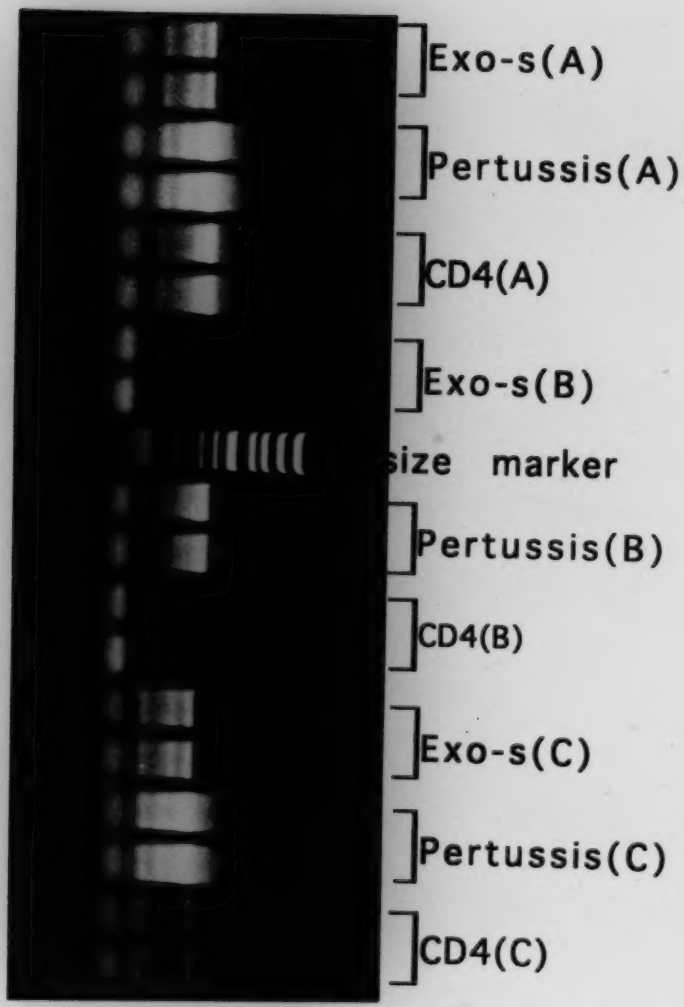

+114
GAG AGT CAG TCC TTC CCA
+120
AAT GCT TTC CCC CTC GTC TCC TGC GAG AGC
+130
CCC CTG TCT GAT AAG...
CTC TCA GTC AGG AAG GGT TTA CAG AAG GGG GAG CAG AGG ACG CTC TCG GGG GAC AGA CTA TTC..
ca gtc agg aag ggt tta cag
cag agg acg ctc tcg ggg gac ag
µc5'(IPCR)P/E
cttaapacgtctctct 5'
EcoRI PstI
µc5'(IPCR)a
µc3'(IPCR)a
g agg acc gtg gac aag tcc ac
XhoI SalI
tctctctcgagtcga
µc3'(IPCR)X/S
c atg tct gac aca ggc ggc
..GAG AGG ACC GTG GAC AAG TCC ACT GGT AAA CCC ACA CTG TAC AAT GTC TCC CTG ATC ATG TCT GAC ACA GGC GGC ACC TGC TAT
...CTC TCC TGG CAC CTG TTC AGG TGA CCA TTT GGG TGT GAC ATG TTA CAG AGG GAC TAG TAC AGA CTG TGT CCG CCG TGG ACG ATA
+600
+610
+620
+628

Figure 24. Location of the I-RT-PCR primers in relation to mouse IgM constant region sequence. The 5' oligos are located in the CH1 region and the 3' oligos in the CH4 region.

Figure 25. Inverted-RT-PCR amplification of mouse heavy chain variable region genes. 1.2% TAE agarose gel with Inverted-RT-PCR reactions involved in isolating D, E and P variable genes. **A, B** and **C** refer to different sets of primers used to amplifiy the variable regions. **C** used μc5'(IPCR)P/E and μc3'(IPCR)X/S (These bands were later used for cloning). **B** used μc5'(IPCR)P/E and μc3'(IPCR)b, and **A** used μc3'(IPCR)X/S and μc5'(IPCR)b. The brightest bands (indicated by arrow) that ran between 0.636-0.812 Kb, where cut out and cloned. For a map of the location of these primers in relation to mouse constant region sequence, see Fig. 23 and 24.


Exo-s(A)
Pertussis(A)
CD4(A)
Exo-s(B)
size marker
Pertussis(B)
CD4(B)
Exo-s(C)
Pertussis(C)
CD4(C)

```
+1                                                  +10
GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG
 E   V   Q   L   V   E   S   G   G   G   L   V   K   P   G   G

                +20                                                 +30
TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT GAC TAT
 S   L   K   L   S   C   A   A   S   G   F   T   F   S   D   Y

                                                +40
TAC ATG TAT TGG GTT CGC CAG ACT CCG GAA AAG AGG CTG GAG TGG GTA
 Y   M   Y   W   V   R   Q   T   P   E   K   R   L   E   W   V

      +50    52  52A 53                                          +60
GCA ACC ATT AGT GAT GGT GGT AGT TAC ACC TAC TAT CCA GAC AGT GTG
 A   T   I   S   D   G   G   S   Y   T   Y   Y   P   D   S   V

                                    +70
AAG GGG CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC AAC CTG TAC
 K   G   R   F   T   I   S   R   D   N   A   K   N   N   L   Y

+80     82  82A 82B 82C 83                                      +90
CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT
 L   Q   M   S   S   L   K   S   E   D   T   A   M   Y   Y   C

                        97 100J 100K 101      *  +103
GCA AGA GAT TTA CTA CGG GGG GCT TAC TGG GGC CAA GGG ACT CTG GTC
 A   R   D   L   L   R   G   A   Y   W   G   Q   G   T   L   V

            *  +113  +114
ACT GTC TCT GCA    GCC AAA
 T   V   S   A      A   K
```

FIGURE 26. DNA and amino acid sequence of D heavy chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 31-35, CDR 2 from 50-65 and CDR 3 from 95-102. CDRs were assigned as in figure 16. * In clone #5 we find a TCC instead of the TCT, which does not change the encoded amino acid. The μ constant region begins with an alanine at position +114.

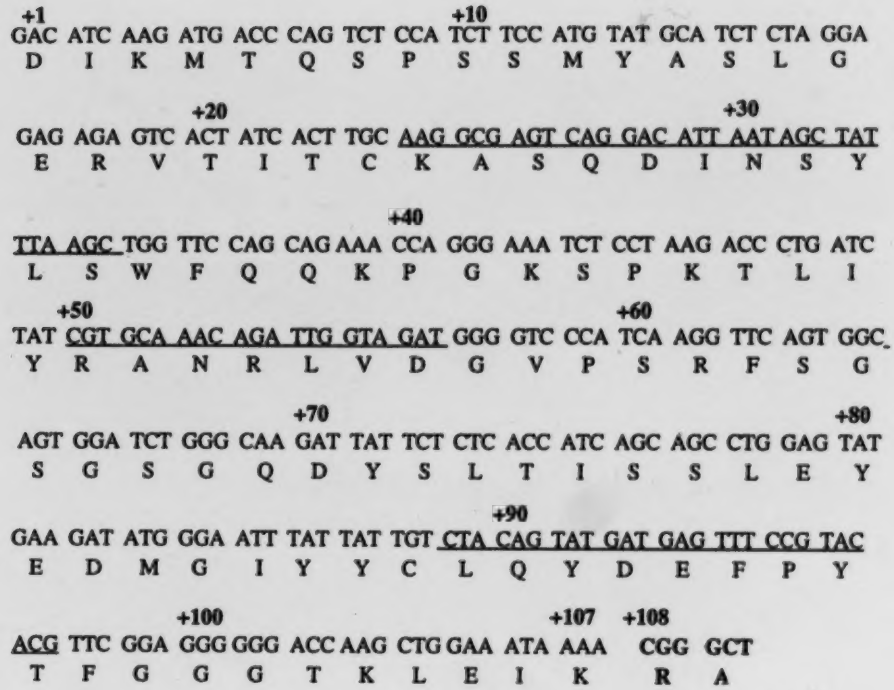

+1
+10
GAC ATC AAG ATG ACC CAG TCT CCA TCT TCC ATG TAT GCA TCT CTA GGA
D I K M T Q S P S S M Y A S L G
+20
+30
GAG AGA GTC ACT ATC ACT TGC AAG GCG AGT CAG GAC ATT AAT AGC TAT
E R V T I T C K A S Q D I N S Y
+40
TTA AGC TGG TTC CAG CAG AAA CCA GGG AAA TCT CCT AAG ACC CTG ATC
L S W F Q Q K P G K S P K T L I
+50
+60
TAT CGT GCA AAC AGA TTG GTA GAT GGG GTC CCA TCA AGG TTC AGT GGC
Y R A N R L V D G V P S R F S G
+70
+80
AGT GGA TCT GGG CAA GAT TAT TCT CTC ACC ATC AGC AGC CTG GAG TAT
S G S G Q D Y S L T I S S L E Y
+90
GAA GAT ATG GGA ATT TAT TAT TGT CTA CAG TAT GAT GAG TTT CCG TAC
E D M G I Y Y C L Q Y D E F P Y
+100
+107
+108
ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT
T F G G G T K L E I K R A

FIGURE 27. DNA and amino acid sequence of D light chain variable region The underlined nucleotides are the CDRs, with CDR 1 from 24-34, CDR 2 from 50-56 and CDR 3 from 89-97. CDRs were assigned as in figure 16. The kappa constant region begins with an arginine at position +108.

```
+1                                                  +10
GAG GTT CAG CTC CAG CAG TCT GGG GCT GAG CTG GCA AGA CCT GGG GCT
 E   V   Q   L   Q   Q   S   G   A   E   L   A   R   P   G   A

                +20                                                 +30
TCA GTG AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTT ACC AGC TAC
 S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y

                                    +40
TGG ATG CAC TGG GTA AAA CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT
 W   M   H   W   V   K   Q   R   P   G   Q   G   L   E   W   I

      +50      52 52A 53                                                +60
GGC GCT ATT TAT CCT GGA AAT AGT GAT ACT AGC TAC AAT CAG AAG TTC
 G   A   I   Y   P   G   N   S   D   T   S   Y   N   Q   K   F

                                +70
AAG GGC AAG GCC AAA CTG ACT GCA GTC ACA TCC GCC AGC ACT GCC TAC
 K   G   K   A   K   L   T   A   V   T   S   A   S   T   A   Y

+80       82  82A 82B 82C 83                                        +90
ATG GAG CTC AGC AGC CTA ACA AAT GAG GAC TCT GCG GTC TAT TAC TGT
 M   E   L   S   S   L   T   N   E   D   S   A   V   Y   Y   C

                                99 100H 100I 100J 100K                  +103
ACA AGA ACC ATT TTA CTA CGG TCC TAT GCT ATG GAC TAC TGG GGT CAA
 T   R   T   I   L   L   R   S   Y   A   M   D   Y   W   G   Q

                                +113 +114
GGA ACC TCA GTC ACC GTC TCC TCA   GCC AAA
 G   T   S   V   T   V   S   S     A   K
```

FIGURE 28. DNA and amino acid sequence of E heavy chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 31-35, CDR 2 from 50-65 and CDR 3 from 95-102. CDR's were assigned as in figure 16. The μ constant region begins with an alanine at position +114.

```
+1                                                  +10
GAT GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA
D   V   V   M   T   Q   T   P   L   S   L   P   V   S   L   G

                +20                                                 27 27A 27B 27C 27D 27E
GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT
D   Q   A   S   I   S   C   R   S   S   Q   S   L   V   H   S

          +30                                                 +40
AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GGC CAG TCT
N   G   N   T   Y   L   H   W   Y   L   Q   K   P   G   Q   S

                    +50
CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA
P   K   L   L   I   Y   K   V   S   N   R   F   S   G   V   P

+60                                                 +70
GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC
D   R   F   S   G   S   G   S   G   T   D   F   T   L   K   I

                +80                 *                   +90
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT
S   R   V   E   A   E   D   L   G   V   Y   F   C   S   Q   S

                                                +100
ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC
T   H   V   P   W   T   F   G   G   G   T   K   L   E   I

+107 +108
AAA  CGG GCT
K    R   A
```

FIGURE 29. DNA and amino acid sequence of E light chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 24-34, CDR 2 from 50-56 and CDR 3 from 89-97. CDRs were assigned as in figure 16. * In clone #7 we find GGT, instead of the GGA in clones #5 and 6, which does not change the encoded amino acid. The kappa constant region begins with an arginine at position +108.

at codon +84 , where clones # 5 and 6 read GGA, whereas clone #7 reads GGT, which does not change the encoded amino acid.

P heavy-chain- See figure 30 for the complete sequence. Three clones were sequenced and all gave the same sequence, except at codon +32 where two clones read TAC and one read CAC, which resulted in a coding change from Y to H. H is highly unusual at this position in V_H genes.

P light-chain- Characterizing this V region sequence was problematic, since the first three clones analyzed all had different DNA sequences. It was unclear which of these corresponded to the expressed light chain, and therefore we went on to sequence additional clones. In all, a total of 15 clones were sequenced, and the sequences could be grouped into four different sets, named I through IV. Representative DNA sequences for each group are shown in figure 31, aligned with the published sequence of an aberrant light chain contributed by the fusion partner (317). The group I sequences (4 out of 15 clones) appeared to be identical to the aberrant light chain, whereas the group III sequences (8 out of 15 clones) appeared to encode a typical light chain V region, which was closely related to but not identical to the aberrant sequence, and which continued 'in frame' into the light chain constant region without any stop codons. The group II sequences (2 out of 15 clones) appeared to be identical to group III in the 5' region, but at about codon 67 they 'switched', and became identical to the aberrant sequence thereafter. Finally, the group IV sequence (1 out of 15 clones) appeared to arise from an unusual splicing event in the group III sequence, which joined codon -5 to codon +55. Because the group II sequences apparently represented the most abundant light chain transcript, and because this was the only sequence obtained which would be capable of encoding a normal light chain V region, we designated it the 'true' P light chain sequence. The deduced protein sequence for this P_L is shown in figure 32, this was the chain which we went on to express as described in chapter 4.

```
+1                                                  +10
CAG GTC CAG CTG CAG CAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT
 Q   V   Q   L   Q   Q   S   G   P   E   L   V   K   P   G   A

                +20                                                 +30       *
TCA GTG AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA AGC TAC
 S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y

                                                +40
TAT ATA CAC TGG GTG AAG CAG AGG CCT GGA CAG GGA CTT GAG TGG ATT
 Y   I   H   W   V   K   Q   R   P   G   Q   G   L   E   W   I

      +50     52  52A                                                +60
GGA TGG ATT TAT CCT GGA GAT GGT AGT ACT AAG TAC AAT GAG AAG TTC
 G   W   I   Y   P   G   D   G   S   T   K   Y   N   E   K   F

                                +70
AAG GGC AAG ACC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC
 K   G   K   T   T   L   T   A   D   K   S   S   S   T   A   Y

+80       82  82A 82B 82C 83                                            +90
ATG TTG CTC AGC AGC CTG ACC TCT GAG GAC TCT GCG ATC TAT TTC TGT
 M   L   L   S   S   L   T   S   E   D   S   A   I   Y   F   C

                                        +100 100A 100I 100J 100K 101
GCA AGG AGA GGT AAC TAC GTA GGG TAC TAT GCT ATG GAC TAC TGG GGT
 A   R   R   G   N   Y   V   G   Y   Y   A   M   D   Y   W   G

                                    +113  +114
CAA GGA ACC TCA GTC ACC GTC TCC TCA   GCC AAA
 Q   G   T   S   V   T   V   S   S   A   K
```

FIGURE 30. DNA and amino acid sequence of P heavy chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 31-35, CDR 2 from 50-65 and CDR 3 from 95-102. CDRs were assinged as in figure 16 * Indicates a nucleotide position which is a TAC (Y) in two clones and a CAC (H) in a third clone; Y is the usual amino acid at this position. The μ constant region begins with an alanine at position +114.

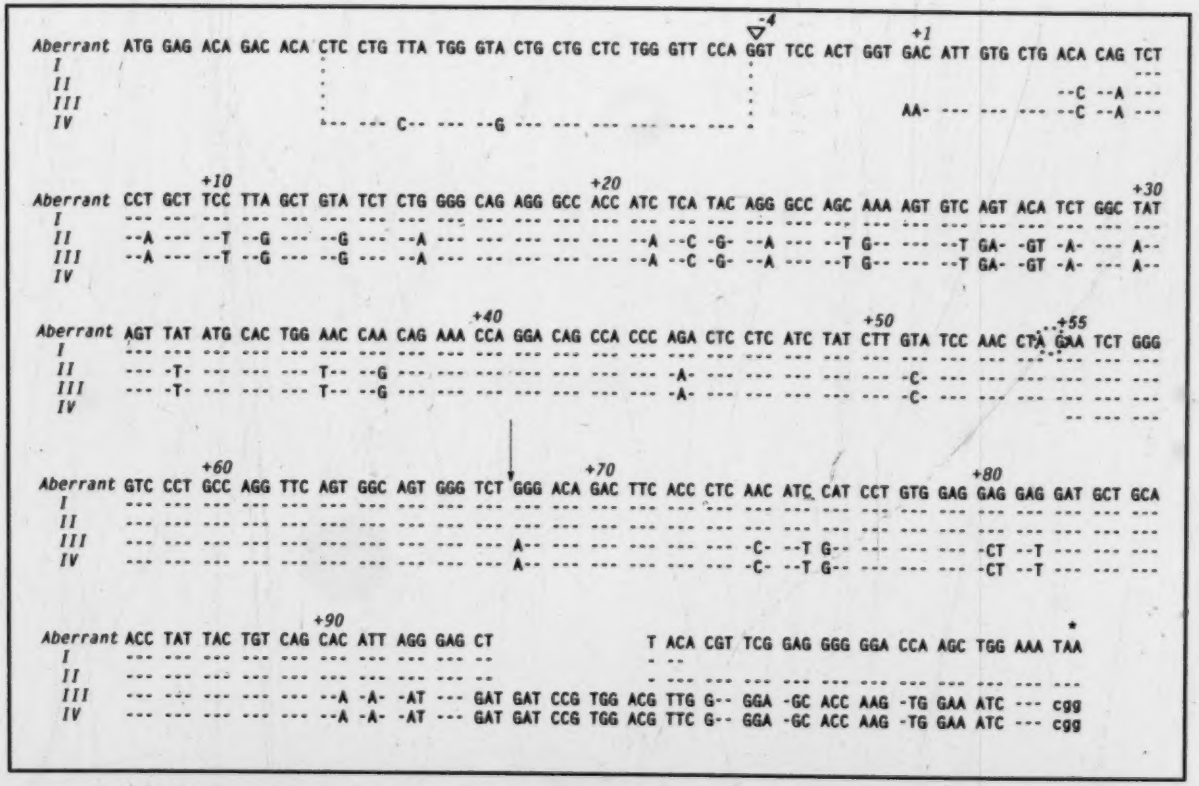

-4
+1
Aberrant ATG GAG ACA GAC ACA CTC CTG TTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC ATT GTG CTG ACA CAG TCT
I
II
III
IV
+10
+20
+30
Aberrant CCT GCT TCC TTA GCT GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TAC AGG GCC AGC AAA AGT GTC AGT ACA TCT GGC TAT
I
II
III
IV
+40
+50
+55
Aberrant AGT TAT ATG CAC TGG AAC CAA CAG AAA CCA GGA CAG CCA CCC AGA CTC CTC ATC TAT CTT GTA TCC AAC CTA GAA TCT GGG
I
II
III
IV
+60
+70
+80
Aberrant GTC CCT GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG GAG GAG GAG GAT GCT GCA
I
II
III
IV
+90
Aberrant ACC TAT TAC TGT CAG CAC ATT AGG GAG CT T ACA CGT TCG GAG GGG GGA CCA AGC TGG AAA TAA
I
II
III
IV

Figure 31. Nucleotide sequences of representative clones from the four sets of PVL clones obtained, in comparison to a published aberrant light chain sequence, produced by the fusion partner (317). Group I is the available sequence of one out of the four clones with sequences exactly identical to that of the aberrant chain. Group II represents one of two clones having group III sequence starting at the 5' end to the ↓ after which they exhibit the aberrant chain sequence. Group III represents a normal VL which occured in 8 of 15 clones, and which was used in the transfection experiments (see Figure 32). Group IV is the sequence of a single clone most probably resulting from aberrant splicing of the group III sequence, fusing the -4 codon to +55 codon. * is a stop codon and the lower case letter below represents the first amino acid of the kappa constant region. The dotted circle indicates a potential splice acceptor site used in the IV region.

```
+1                                                  +10
AAC ATT GTG CTG ACC CAA TCT CCA GCT TCT TTG GCT GTG TCT CTA GGG
 N   I   V   L   T   Q   S   P   A   S   L   A   V   S   L   G

                +20                                                 27  27A 27B 27C 27D 28
CAG AGG GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT GTT GAT AGT TAT
 Q   R   A   T   I   S   C   R   A   S   E   S   V   D   S   Y

     +30                                                +40
GGC AAT AGT TTT ATG CAC TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC
 G   N   S   F   M   H   W   Y   Q   Q   K   P   G   Q   P   P

                +50                                                 +60
AAA CTC CTC ATC TAT CTT GCA TCC AAC CTA GAA TCT GGG GTC CCT GCC
 K   L   L   I   Y   L   A   S   N   L   E   S   G   V   P   A

                                                +70
AGG TTC AGT GGC AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC ATT GAT
 R   F   S   G   S   G   S   R   T   D   F   T   L   T   I   D

                +80                                                 +90
CCT GTG GAG GCT GAT GAT GCT GCA ACC TAT TAC TGT CAG CAA AAT AAT
 P   V   E   A   D   D   A   A   T   Y   Y   C   Q   Q   N   N

                                                +100
GAG GAT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC
 E   D   P   W   T   F   G   G   G   T   K   L   E   I

+107  +108
AAA   CGG  GCT
 K     R    A
```

FIGURE 32. DNA and amino acid sequence of P light chain variable region. The underlined nucleotides are the CDRs, with CDR 1 from 24-34, CDR 2 from 50-56 and CDR 3 from 89-97. CDRs were assigned as in figure 16. The kappa constant region begins with an arginine at position +108.

CHAPTER IV

EXPRESSION AND CHARACTERIZATION OF CHIMERIC MOUSE/HUMAN IgG3 AND IgG1 ANTIBODIES

A. *Introduction*

Once the required mouse V-region cDNAs were obtained we proceeded to engineer mouse/human chimeric antibodies. The overall strategy involved insertion of the mouse V-region coding elements into eucaryotic expression vectors containing human C-region coding elements, transient transfection of these plasmids into COS cells, harvesting of resulting supernatants, and analysis of these by sandwich and competitive ELISA assays and by western blotting. This chapter describes in detail the various cloning/expression strategies undertaken, and provides evidence that: 1) expected chimeric antibodies were eventually obtained in all cases, and 2) in most cases binding to pili was at least comparable to that for the parental mouse monoclonal antibodies, and in some cases it was actually better.

Initially we were interested in expressing human IgG3 chimeric antibodies, since it was felt that these would be most efficient at complement activation. Because no eucaryotic expression vector for this antibody isotype was available, we began by constructing the necessary vector. In fact two different versions of the γ3 vector were eventually constructed, which differed in the presence or absence of an intron u *Pst*ream of the V region coding segment. These two vectors were called simply 'version γ3.a' and 'version γ3.b'. In parallel similar 'version k.a ' and 'version k.b ' human kappa expression plasmids were also constructed. All 4 antibodies (B, D, E, P; see last chapter) were expressed successfully in the 'b version' γ3 and kappa vectors. Later on in the project when it became apparent that IgG3 chimeric antibodies were not always as

stable as desired, we went on to express all 4 antibodies as IgG1 mouse/human chimeras. This was accomplished using an expression plasmid obtained from our collaborators, and the resulting constructs were termed 'version γ1.c'. Availability of chimeric antibodies of both isotypes allowed us to compare binding for immunoglobulins with identical V-regions but different CH-regions.

B. ***Construction of γβ.a and κ.a expression vectors***

As originally conceived, we wished the version 'a' vectors to contain the following elements: 1) a high copy number origin of replication (e.g. pUC) to facilitate rapid production of large amounts of plasmid DNA for COS cell transfections, 2) an M13 or similar origin of replication so that single stranded molecules could be made if necessary, 3) a promoter which would work well in transiently transfected COS cells, so that supernatants would contain workable levels of recombinant antibodies within a few days of transfection.

In order to be secreted, the recombinant antibody chains require a leader sequence. This could be supplied as the leader/variable, a unique sequence which would be present at the 5' end of each full length cDNA, or alternately the leader sequence could be 'generic', and be supplied by the expression vector itself. We chose the latter strategy, since this is simpler, leads to more uniform expression, and does not require full length cDNAs for each and every V-region. Instead of fusing the 'generic' leader coding segment directly to the V-region coding segment at the DNA level (which involves creating restriction sites in the coding segments, and hence alters the protein) we decided that our 'version a' vectors should contain an intron between the L and V. This approach means that the precise 'fusion' of the L and V coding elements occurs at the mRNA level, with the disappearance of all restriction sites used to clone the V-region into the intron. By a similar argument, fusion of the appropriate V region with the human CH or CL could be accomplished at the DNA level or the RNA level. Following

the same reasoning as above, in the 'version a' vectors fusion of the V and C coding segments was also accomplished by utilizing an intron between these two elements.

The backbone of the 'version a' vectors (named pJHE.s) was constructed from the plasmid pBluescript SK$^-$ as summarized in figure 33. The creation of pJHE.s involved two basic steps, the elimination of unwanted restriction sites in pBluescript SK$^-$, and insertion of the of the *Hind*III/*Eco*RI fragment of pJFE14. pBluescript SK$^-$ supplied the pUC and M13 origins, and pJFE14 the SRα promoter/enhancer and the polycloning site (see figures 12 and 33).

The leader sequence and downstream intron for the 'version a' vectors was taken from the expression vector YP04 (Brian Seed, Harvard University). This leader sequence is identical to that of human T-cell surface glycoprotein (T1/Leu1) (318), and the intron is an IgG intron located between the CH1 domain and the hinge region. The desired DNA from YP04 was removed using restriction enzymes, modified at the 5' end using oligonucleotides, and inserted into pJHE.s as shown in figure 34. The resulting vector was named pJL.

In order to complete the 'version a' vectors, it was necessary to insert either the genomic fragment for the human C kappa chain, or the genomic fragment for the human Cγ3 into pJL. The necessary constant region genomic clones were obtained from ATCC (clone pHuCk, ATCC # 59173 for kappa, and clone pSH2-γ3, ATCC # 59621 for gamma). In order to construct pJLk.a the 2.5 kb *Eco*RI genomic fragment from pHuCk was cloned into the *Eco*RI site of pJL (figure 35). In order to construct pJLγ3.a the 2.55 kb *Hind*III-*Sph*I genomic fragment from pSH2-γ3 was modified at each end using oligonucleotides, and cloned into the *Eco*RI site of pJL (figure 36). The pJLγ3.a and pJLk.a vectors are also illustrated in figures 37 and 38, which demonstrates the precise details of the splice donor and acceptor sites, and their relationship to the coding regions of the L, V, and C exons (see below for more details).

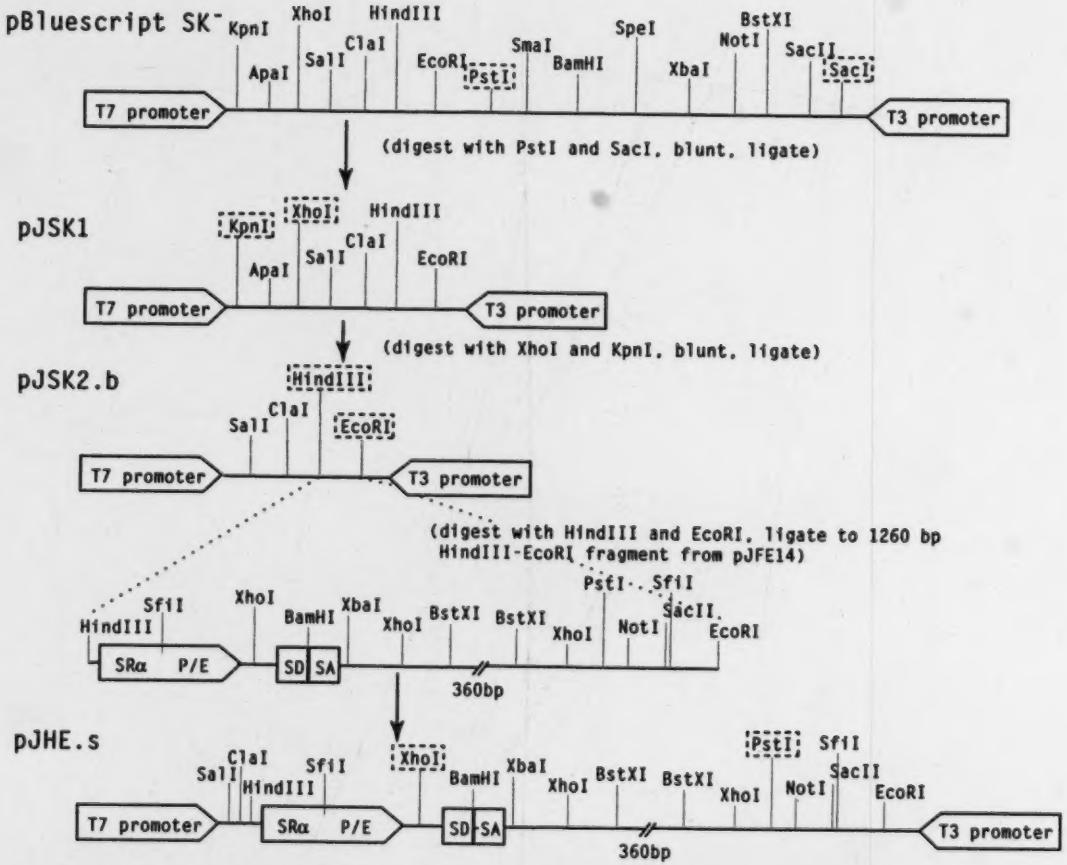


Figure 33. Steps in construction of pJHE.s from pBluescript SK⁻ and pJFE14. SRα is an SV40 derived promoter-enhancer element; SD and SA refer to SV40 derived splice donor and splice acceptor elements.

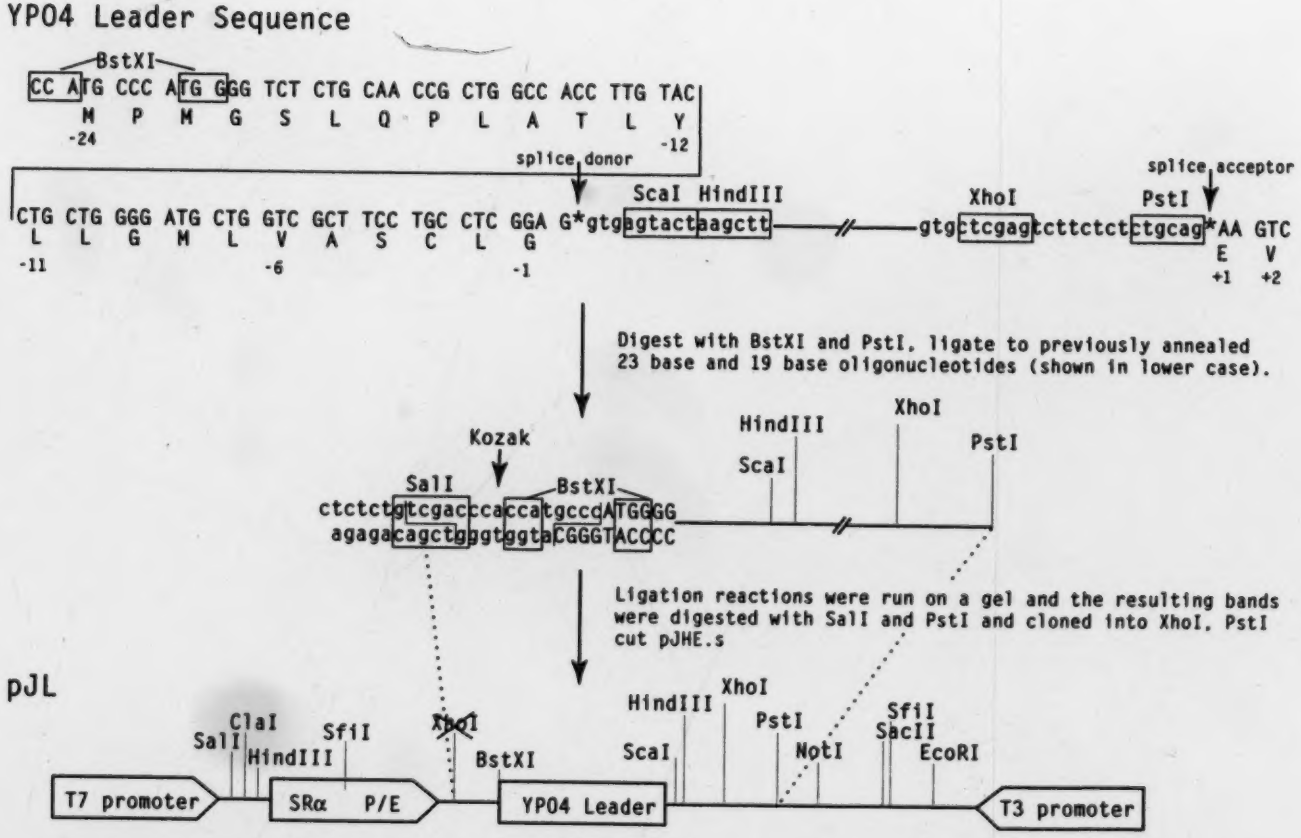


Figure 34. Construction of pJL by insertion of YPO4 Leader sequence into pJHE.s (bottom of Figure 33). Note that *Sal*I and *Xho*I 'sticky ends' are compatible, but that both sites are destroyed upon ligation.

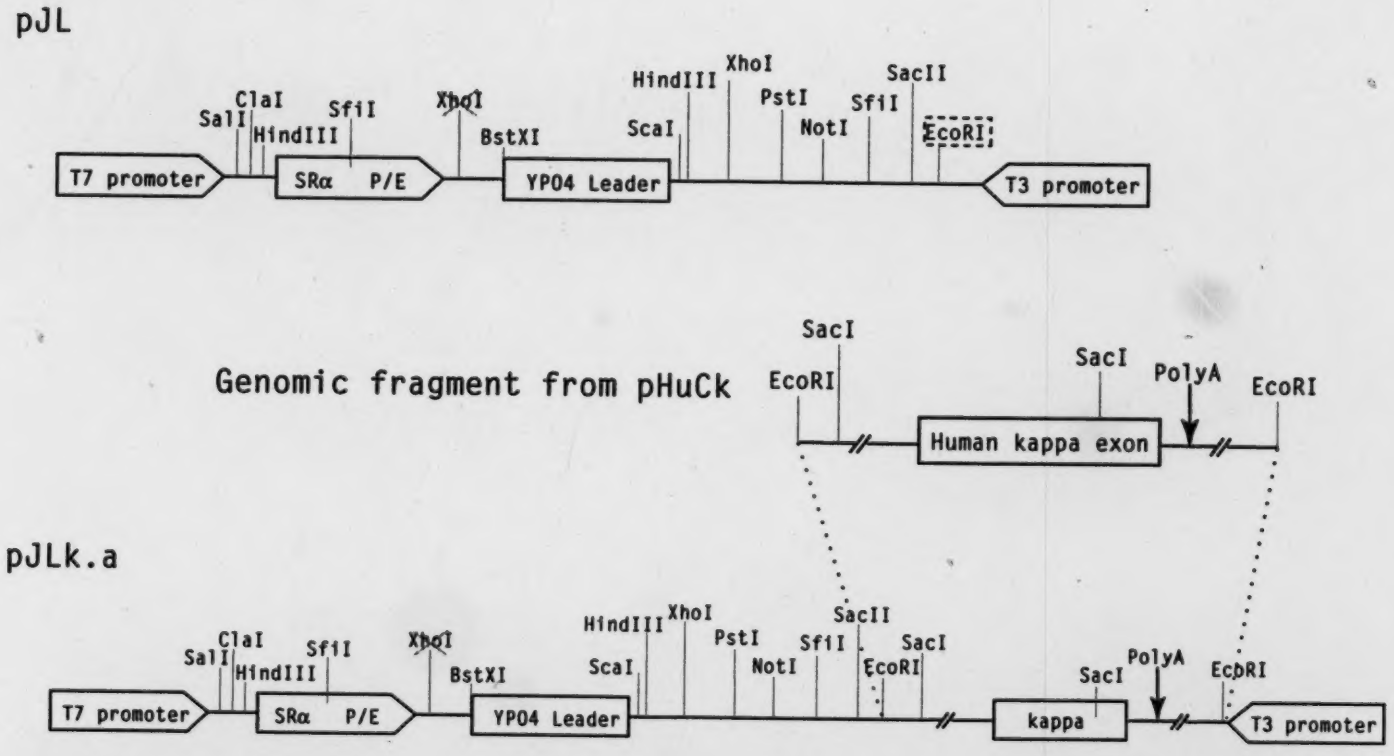


Figure 35. Construction of pJLk.a by ligation of 2.5 kb Ck *Eco*RI genomic fragment into*Eco*RI cut, dephosphorylated pJL. The insert was mapped by restriction enzyme digests for correct orientation.

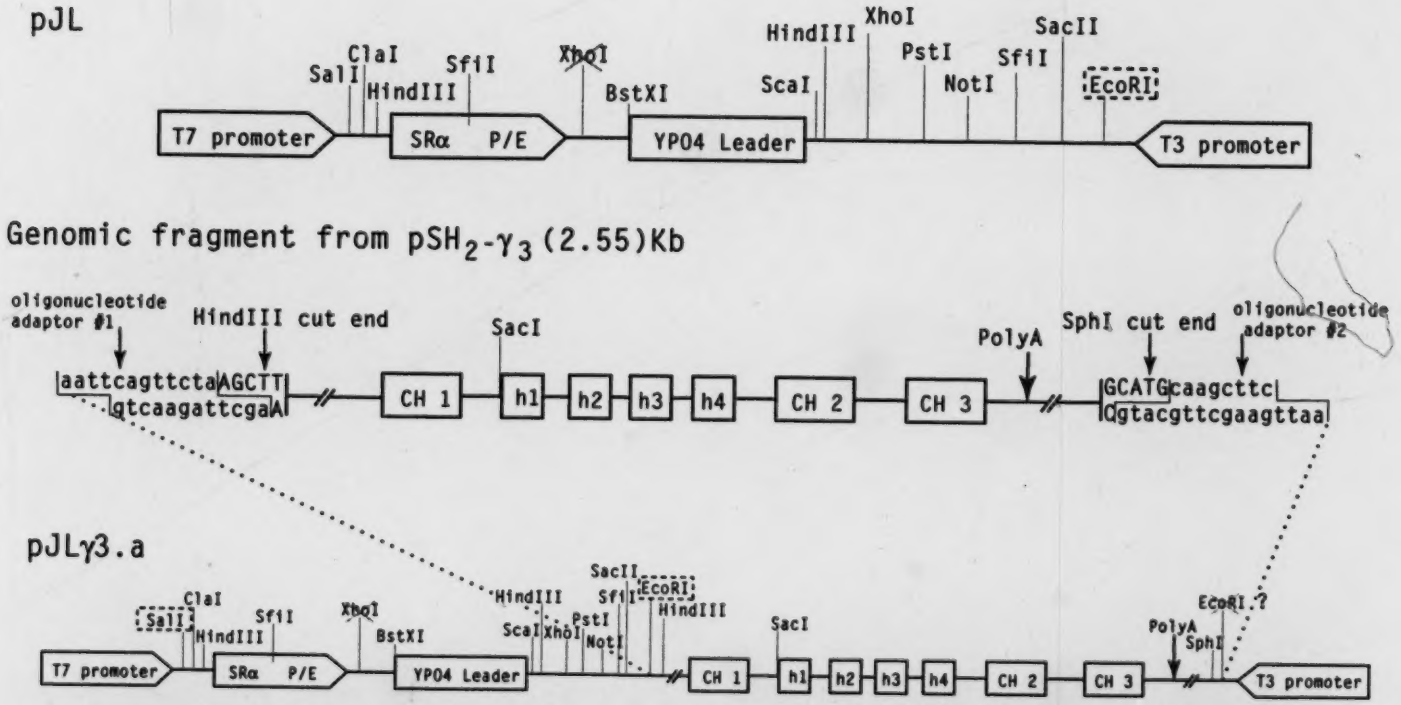


Figure 36. Construction of pJLγ3.a by simultaneous ligation of $C\gamma_3$ 2.55 kb *Hind*III-*Sph*I genomic fragment to two different phosphorelated *Eco*RI linker-adaptors (shown in lower case), Cγ3 with ligated linkers was then digested with *Eco*R1, ran on a gel and the band was extracted. This was followed by ligation into *Eco*RI cut, dephosphorylated pJL. The insert was mapped with restriction enzyme digests for correct orientation. Oligonucleotide adaptor #1 consists of H/E -UP and H/E-DN. Oligonucleotide adaptor #2 consists of *SphI*/*Eco*RI 8 and *SphI*/*Eco*RI 16. *Eco*RI?--due to cloning strategy, we could have had a number of linkers added at this end.

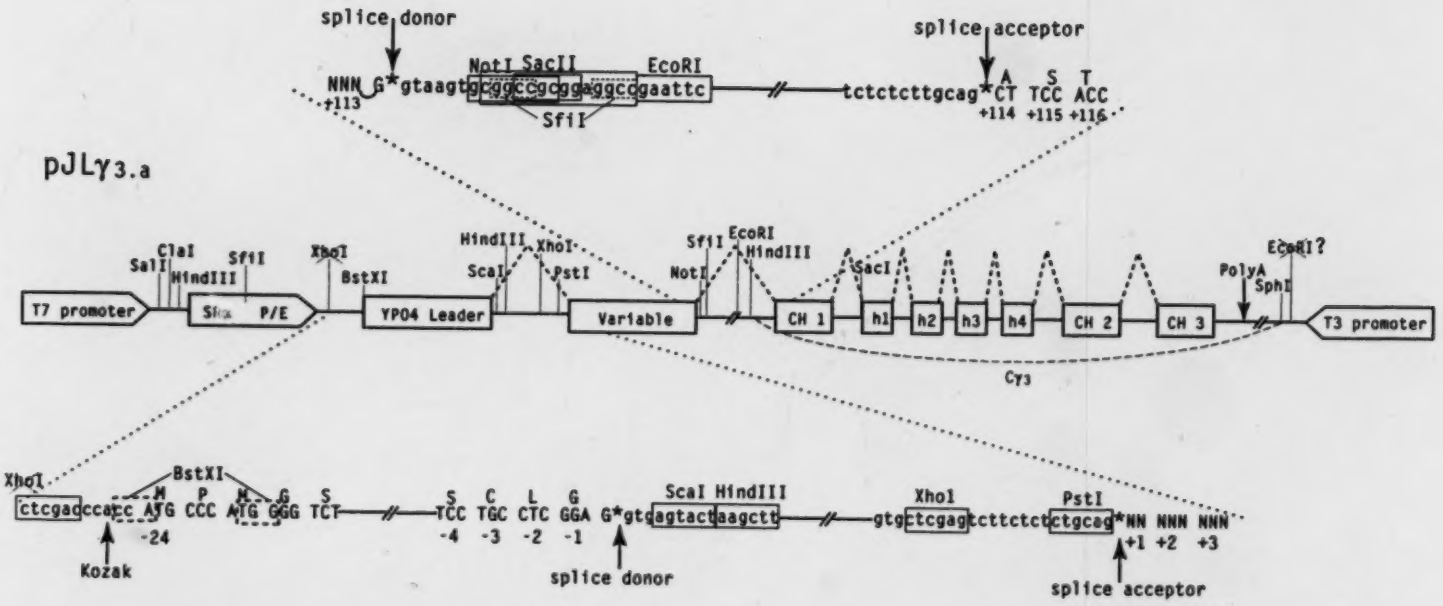


Figure 37. Details of splice donors and splice acceptors both upstream and downstream of any cloned variable region exon placed in pJLγ3.a . Variable region exons were generated from cDNAs by PCR, and were cloned using *Xho*I or *Pst*I as 5' sites, and *Not*I as the 3' site. In all cases care was taken to preserve the splice acceptor and splice donor sequences exactly as shown.

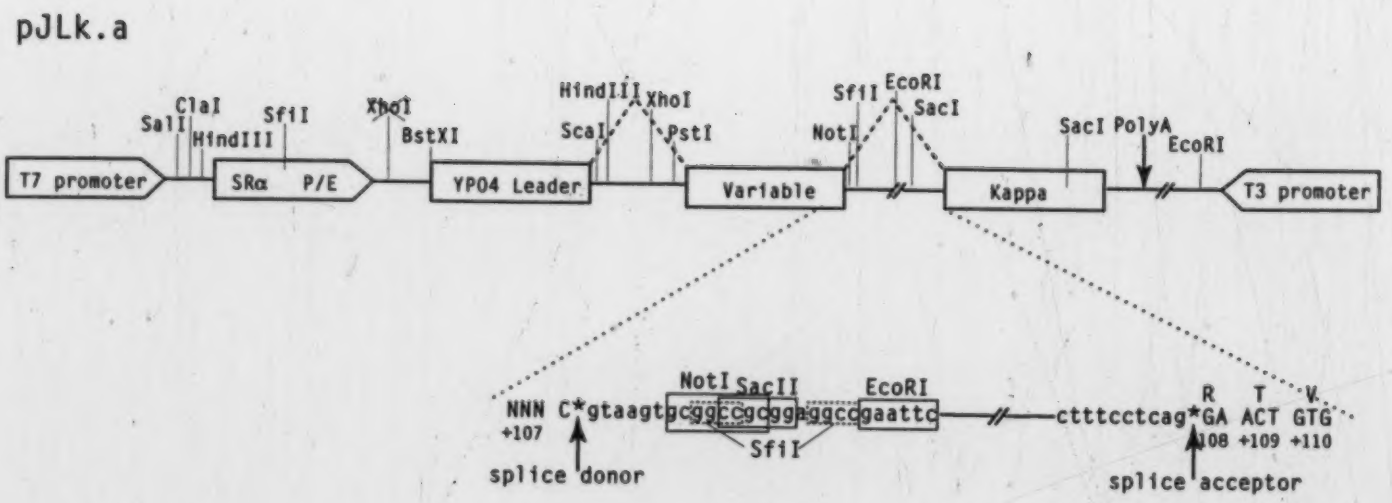


Figure 38. Details of splice donor and splice acceptor sites downstream of any cloned variable region exon placed in pJLk.a. Upstream splice donor and splice acceptor sites are the same as in Figure 37, as are cloning sites and strategy.

C. ***Cloning and expression of the B [PK3B] antibody V-regions using the γ3.a and k.a expression vectors***

Initially we cloned the B heavy chain variable region into pJLγ3.a and the B light chain variable region into pJLk.a. The variable regions were cassetted into these vectors using the *Xho*I site as the 5' cloning site and *Not*I as the 3' cloning site. The variable genes were amplified from the original SK$^-$ clones using PCR primers designed especially for this purpose. In designing these PCR primers three factors were taken into consideration: 1) conservation of functional splice donor and acceptor sites, 2) promotion of proper cleavage of the leader peptide, and 3) generation of the proper codons at the L/V and V/C junctional regions.

According to Mount (319), the consensus splice donor sequence is AG-gt, and the consensus splice acceptor site is ag-N, where the lower case letters represent bases which are absolutely required at the intron boundaries, and the upper case letters represent the most common bases found at the margins of the exons (N represents any nucleotide).

Cleavage of the leader peptide occurs between the -1 residue (which is fixed as glycine in the case of the YP04 leader) and the +1 residue. The choice of the amino acids in the +1 and +2 positions is apparently important for proper cleavage of the leader peptide. Von Heijne (320,321) has tabulated the frequency of amino acids found in and around signal cleavage sites, including the +1 and +2 positions. Throughout the expression cloning experiments described in this chapter we were careful to design PCR primers so that the +1 and +2 triplets encoded residues which occurred at a high frequency (according to Von Heijne), rather than simply using the +1 and +2 codons found in the wild-type V-region sequence. Because in the 'version a' vectors the YP04 leader sequence contributes a 'G' as the first base of the +1 codon, our choices of the +1 amino acid were limited (figure 37), but could still be made consistent with Von Heijne's 'rules' (320,321).

According to Kabat (118), the first C-region exon of human Cγ3 begins with the second base of codon +114 (figure 37). The most frequent amino acid at position +114 is alanine (e.g. A), encoded by GCT. Therefore the first 'G' of this codon must be included in the 3' primer used to PCR a VH coding region. Beyond this the variable gene can be

maintained exactly as in the original mouse sequence. Likewise the human kappa constant region exon begins at codon +108, but encodes only the last two bases of this codon (figure 38). The most frequent amino acid at position +108 is arginine (e.g. R), encoded by CGA. Therefore this 'C' must be included in any 3' primer used to PCR a VL coding region.

As a specific example, the primers used to PCR amplify and clone the B antibody VH region into the pJLγ3.a vector are shown in figure 39. The primers added 5' and 3' restriction sites, as well as the necessary 5' splice acceptor and 3' splice donor sites. A similar strategy was used to clone the B VL region into pJLk.a, using primers 3B5' K-2 and 3B3' K-3 listed in table 4. The BVH and BVL containing expression plasmids, as well as the 'empty' pJLγ3.a and pJLk.a plasmids were grown up in bulk, purified over Qiagen columns, and used to transfect monolayers of COS cells as described in Chapter 2. After 96 hours of culture the resulting supernatants were collected and assayed by sandwich ELISA to detect secreted antibody. Surprisingly, supernatants from cells transfected with the 'empty' pJLγ3.a and pJLk.a plasmids gave a signal about 8 fold higher than background (e.g. mock transfected COS cell supernatants), and almost the same as that from cells transfected with the BVH and BVL containing expression plasmids. This experiment was repeated again with a similar result. When supernatants from cells transfected with the 'empty' pJLγ3.a and pJLk.a plasmids were analyzed by western blot (probed with goat anti-human IgG H + L), two major bands at ≈19 kD and ≈45 kD were seen. These bands persisted even when constructs containing V-regions were transfected along with new high molecular weight bands (data not shown).

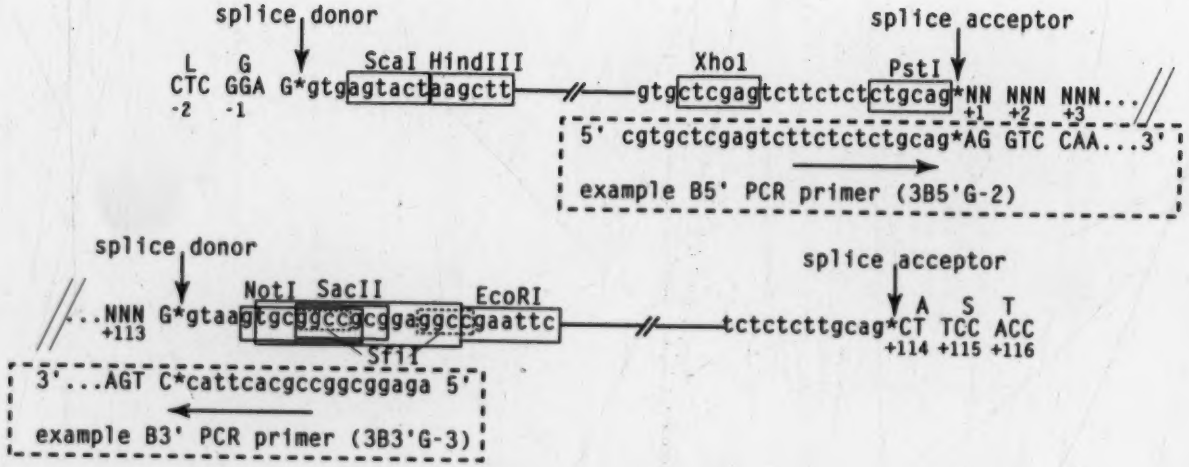


Figure 39. Example PCR primers used to generate the B heavy chain V region exon which was then cloned into pJLγ3.a using the *Xho*I and *Not*I sites. The template for the PCR was the cloned B heavy chain cDNA. The resulting +1 codon of B is GAG, although the original cloned gene started with a CAG (Q→ E).

The results obtained with the 'empty' pJLγ3.a and pJLk.a plasmids suggested that they were inducing the secretion of heavy and light chain constant regions, without any V-regions. When I reviewed my cloning strategy (figure 37), it became immediately apparent that the YP04 leader exon would be able to splice directly to the first exon of Cγ3, and thus generate and in-frame transcript. The same direct splicing of the YP04 leader exon to the Ck exon was also possible (figure 38). Thus, even if V-region exons were inserted into the version 'a' vectors, there was little assurance that significant amounts of the expected chimeric antibodies would be made and that the analysis would not be complicated by the fact that truncated ('V-less') proteins were present. For this reason we proceeded to construct a new series of expression vectors (the 'version b' vectors) designed to insure that any inserted V-region coding segment would be included in the final spliced mRNA.

D. ***Construction of γ3.b and k.b expression vectors***

The 'version b' vectors were very similar to the 'version a' vectors described above, but the intron located between the generic YP04 leader segment and any cloned V region was eliminated. Removal of the leader/variable intron meant that a 5' cloning site had to be created either in the 5' coding sequence of the variable, or in the 3' coding sequence of the leader. To give maximum flexibility in the choice of the +1 and +2 amino acids, which remain in the mature antibody and which can influence cleavage of the leader peptide (see above), we chose to introduce a restriction site (*Bam*HI) in the -4, -3 codons of the leader sequence (figures 40 and 41).

Details of the construction of the 'b version' vectors (pJLγ3.b and pJLk.b) are shown in figure 40. The essential idea behind the construction was to remove the SRα promoter, YP04 leader, and downstream intron from a 'version a' vector to a more convenient intermediate plasmid (pHAS). This facilitated removal of the YP04 leader and downstream intron, and it's direct replacement with a shorter, intronless version of the YP04 coding segment which had been modified by PCR to include a *Bam*HI site in

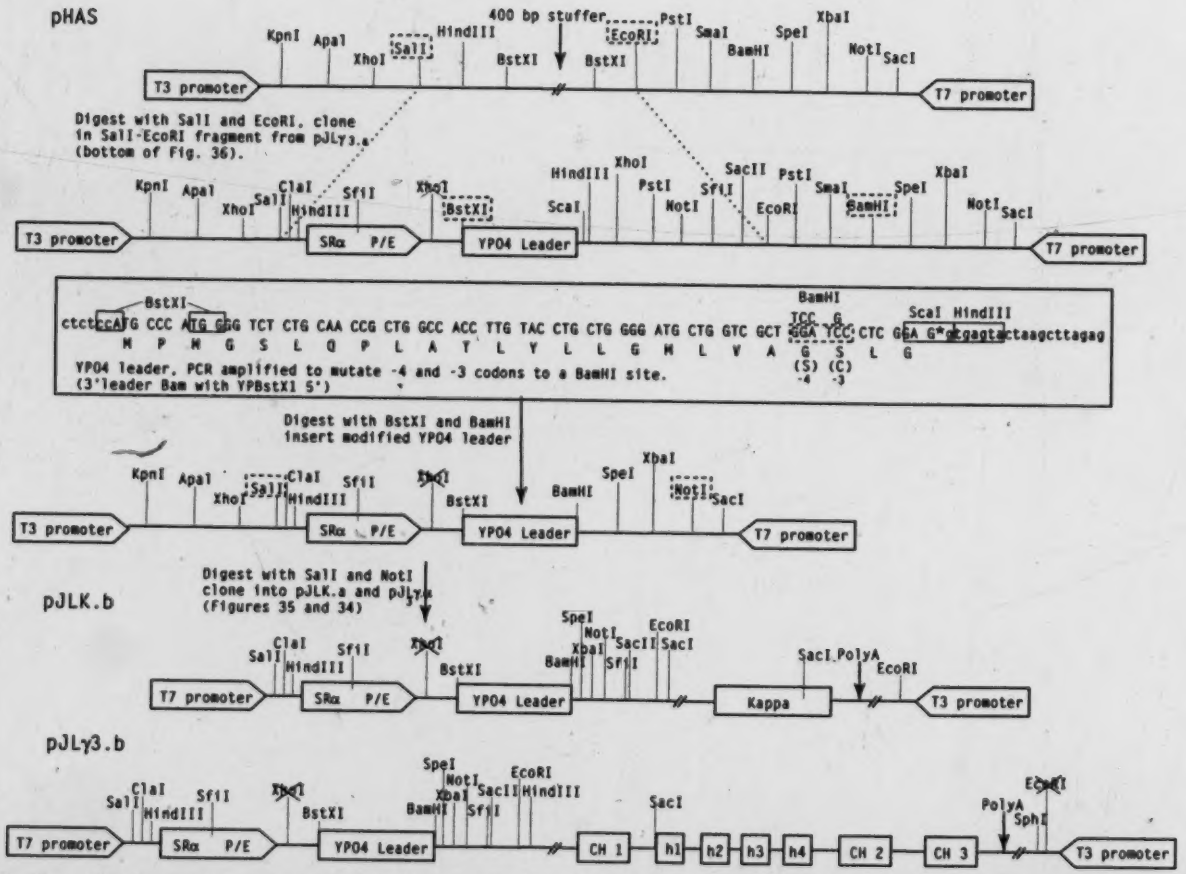


Figure40. Steps in the construction of pJLK.b and pJLγ3.b. pHAS is a modified pBluescript KS ⁻plasmid obtained from Dean Smith in Dr. Elliott's laboratory. PCR primers used to modify the YPO4 leader sequences were [YP*Bst*XI 5'] and [3' leader *Bam*HI] listed in Table 4. Original coding nucleotides are shown above the *Bam*HI sequence, and original encoded amino acids shown below in brackets.

the -4 and -3 codons. The promoter plus shortened YP04 leader was then placed back in the original 'version a' vectors (both γ3 and k) to create the 'version b' vectors. The precise details of the YP04 leader/variable fusion and the downstream splice donor and acceptor sites is shown in figure 41. Essentially the intron between the V and C segments is identical to that present in the 'version a' vectors.

E. *Cloning of B [PK3B], D [CD4-TT], E [Ex0-s], and P [18B-13-41] into γ3.b and k.b expression vectors*

To clone the variable genes into the 'version b' vectors, 5' primers were made which contained a *Bam*H I restriction site (*Bgl*II for PVL), as shown in figures 42 and 43 (complete primer sequences are listed in table 4). For all heavy chain V regions, the +1 codons were altered to glutamine (Q) if they did not already occur in the original antibody (figure 42). For all light chain V regions, the +1 codons were altered to aspartic acid (D), and the +2 codons to isoleucine (I) if they did not already occur in the original antibody (figure 43). These coding changes were made for three reasons: 1) so that the +1 and +2 residues corresponded to those which occur most frequently in known VH's or VL's, 2) so that cleavage of the leader peptide was likely according to the data of Von Heijne (see above), and 3) so that cleavage and secretion would be as uniform as possible between the various antibodies, considering that each of them were using the same generic leader peptide. These 5' primers were used in combination with 3' primers containing *Not*I restriction sites, designed using the same principles as described above for the 3' primers used to clone the B antibody V regions into the 'version a' vectors. The PCR fragments were digested with *Bam*HI (or *Bgl*II) and *Not*I, and cassetted into the appropriate heavy or light chain 'version b' vectors.

F. *Expression of B [PK3B], D [CD4-TT], E [Ex0-s], and P [18B-13-41] IgG3 chimeric antibodies*

To obtain chimeric IgG3 immunoglobulins for antibodies B, D, E, and P, γ3.b constructs containing each of the various VH segments were co-transfected into COS

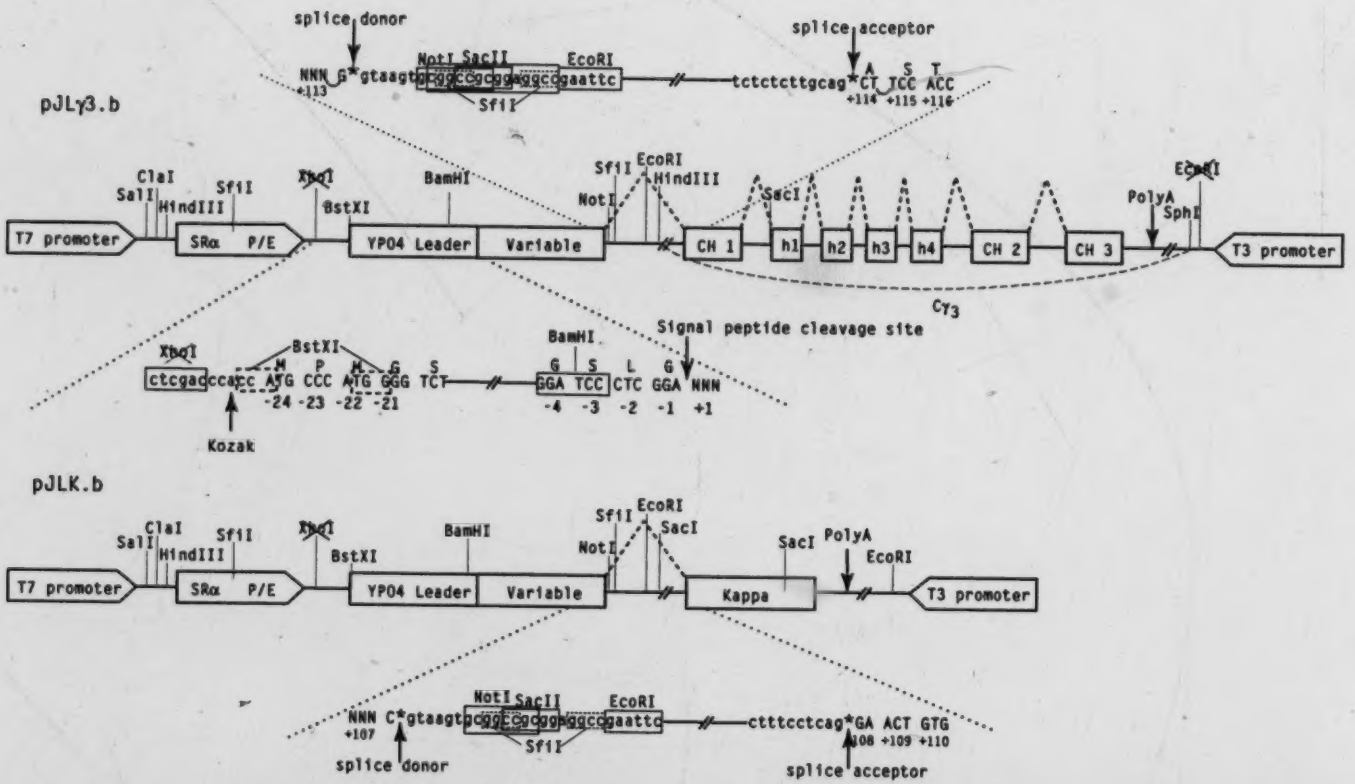
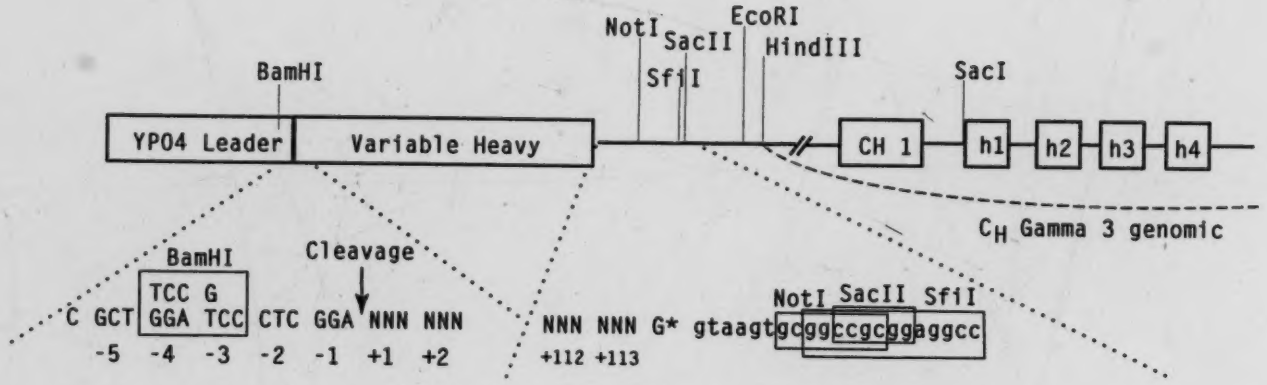
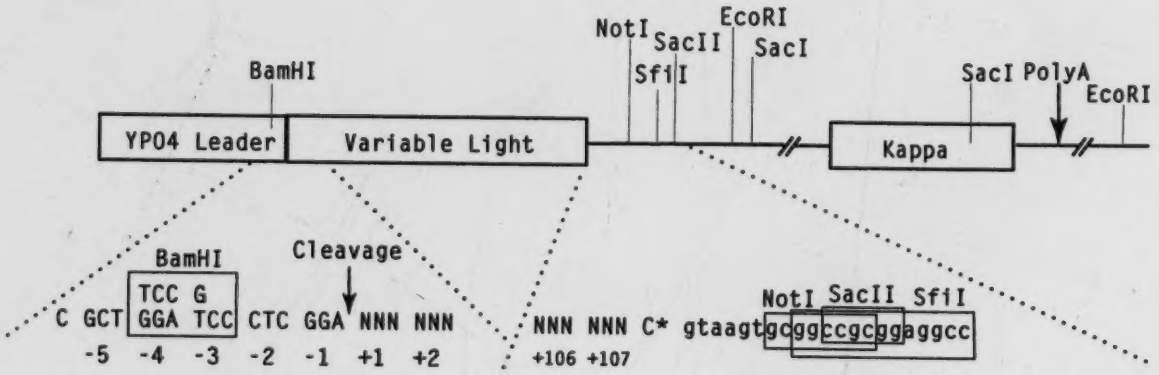


Figure 41. Strategy for cloning variable region genes into pJLγ3.b and pJLK.b, showing details of fusion point with Leader sequence as well as downstream splice donor and splice acceptor sites. V region genes were engineered by PCR and cloned as *Bam*HI (or *Bgl*II) -*Not*I fragments, keeping the splice donor site exactly as shown. The Leader sequence V-region fusion is identical in both vectors, and hence is shown only once.



5' primers (name)	BamHI	+1	+2	3' primers (name)	+113	NotI
BV_H (3BG5'Bam)	C GCT GGA TCC CTC GGA	CAG	GTC...	BV_H(3B3'G1-3)	... TCC TCA G *	gtaagtgcggccgc
DV_H (CDμ5'Bam)	C GCT GGA TCC CTC GGA	GAA(E)* CAA(Q)	GTG...	DV_H(CDμ3'-2)	... TCT GCA G *	gtaagtgcggccgc
EV_H (Exoμ5'Bam)	C GCT GGA TCC CTC GGA	GAG(E)* CAG(Q)	GTT...	EV_H(P+E3'μ)	... TCC TCA G *	gtaagtgcggccgc
PV_H (Perμ5'Bam)	C GCT GGA TCC CTC GGA	CAG	GTC...	PV_H(P+E3'μ)	... TCC TCA G *	gtaagtgcggccgc

Figure 42. Details of primers used to create the various VH/Cγ3 constructs. * is the original V-region sequence. Changed all +1 to Q if they were not already, since they were the most common +1 residues in VH chains, and also likely to give good cleavage of the leader peptide.



5' primers (name)		BamHI			+1	+2	3' primers (name)		+107	NotI
BV_L(3Bκ5'Bam)	C GCT	GGA TCC	CTC GGA	GAT	ATC...		BV_L(3B3'κ-3)	... ATC	AAG C *	gtaagtgcggccgc
DV_L(CDκ5'Bam)	C GCT	GGA TCC	CTC GGA	GAC	ATC...		DV_L(CDκ3-2)	... ATA	AAA C *	gtaagtgcggccgc
EV_L(Exoκ5'Bam)	C GCT	GGA TCC	CTC GGA	GAT	GTT(V)* ATT(I)...		EV_L(P+E3'κ)	... ATC	AAA C *	gtaagtgcggccgc
		BglII		AAC(N)*						
PV_L(Perκ5'BglII)	C GCT	AGA TCT	CTC GGA	GAT(D)	ATT...		PV_L(P+E3'κ)	... ATC	AAA C *	gtaagtgcggccgc

Figure 43. Details of the primers used to create the various VL /κ constructs containing an internal intron. *The original sequence. We changed all +1 to a D if they were not already and all +2 were changed to an I if they were not already. There are common residues at these positions and they are most likely to give good cleavage of the leader peptide.

cells along with the corresponding V_L segments in the k.b expression vector. Plasmids were grown up in bulk, purified over Qiagen columns or by the modified alkaline preparation method, and used to transfect monolayers of COS cells as described in Chapter 2. Supernatants were collected and assayed by sandwich and competitive ELISA , and by western blotting.

G. *Cloning of B [PK3B], D [CD4-TT], E [Ex0-s], and P [18B-13-41] heavy chain V segments into a γ1.c expression vector*

We went on to generate chimeric mouse/human IgG1 antibodies using the vector pSRαSD5/γ1 (figure 44), which was obtained from D. Denny and C. Okada (Stanford). Motivation for these experiments was three-fold: 1) with the ready availability of pSRαSD5/γ1, cloning of the V_H genes would be a simple and rapid operation, 2) production of human chimeric antibodies with identical V regions but two different possible C_H regions would allow us to observe the effect of altering the C region on antigen binding, and 3) we anticipated that the IgG1 molecules would be more stable, and thus perhaps more useful in long-term experiments.

A more detailed drawing of the features of pSRαSD5/γ1 is shown in figure 45. In comparison to the 'version a' and 'version b' vectors described above, this vector (which we label 'version c') has no introns what-so-ever within the chimeric antibody message. Instead, as single intron (derived from the SV40 virus) is located upstream of any inserted cDNA. cDNAs encoding a V_H are placed in frame with a cDNA segment encoding human Cγ1, which is provided by the vector. Fusion of the V_H and Cγ1 coding elements is via a *SalI* site which spans codons +114, +115, and +116 of the human Cγ1 cDNA.

To create the various 'version c' expression plasmids, the YP04 leader segment fused to the various V_H coding elements (as already existed in the corresponding 'version b' vectors) were amplified by PCR to add a 5' *Not*I site and a 3' *Sal*I or *Xho*I site, as shown in figures 46 and 47. The 5' primer was designed to removed the first

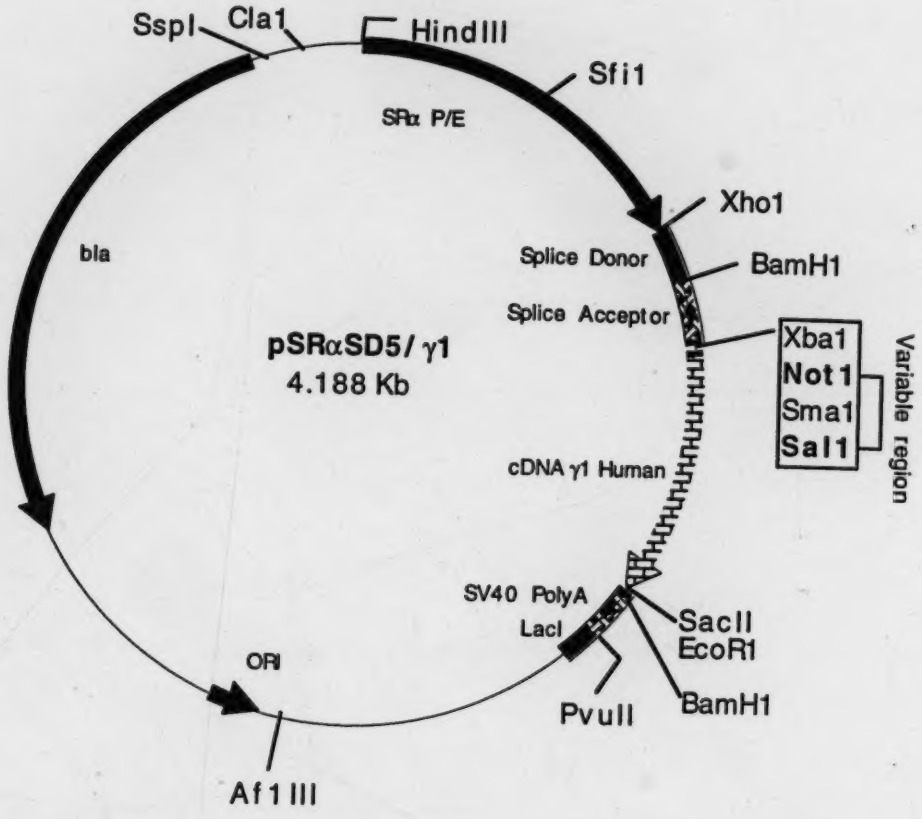


Figure 44. Map of pSRαSD5/γ1.

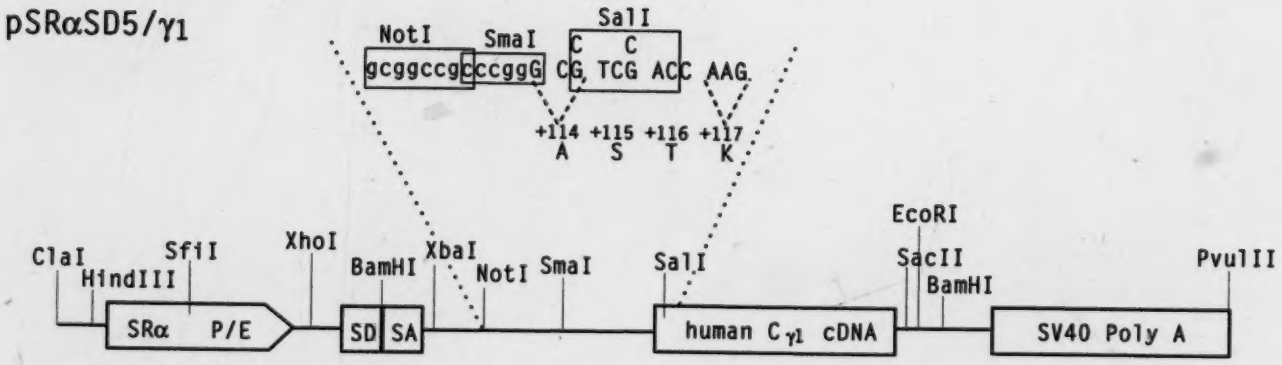


Figure 45. Details of pSRαSD5/γ_1 (from Dan Denny and Craig Okada, Stanford University). The 5' end of Cγ was mutated by Okada to add a *Sal*I site, without altering the encoded protein (original nucleotides are listed above). SD and SA refer to SV40-derived splice donor and splice acceptor elements.

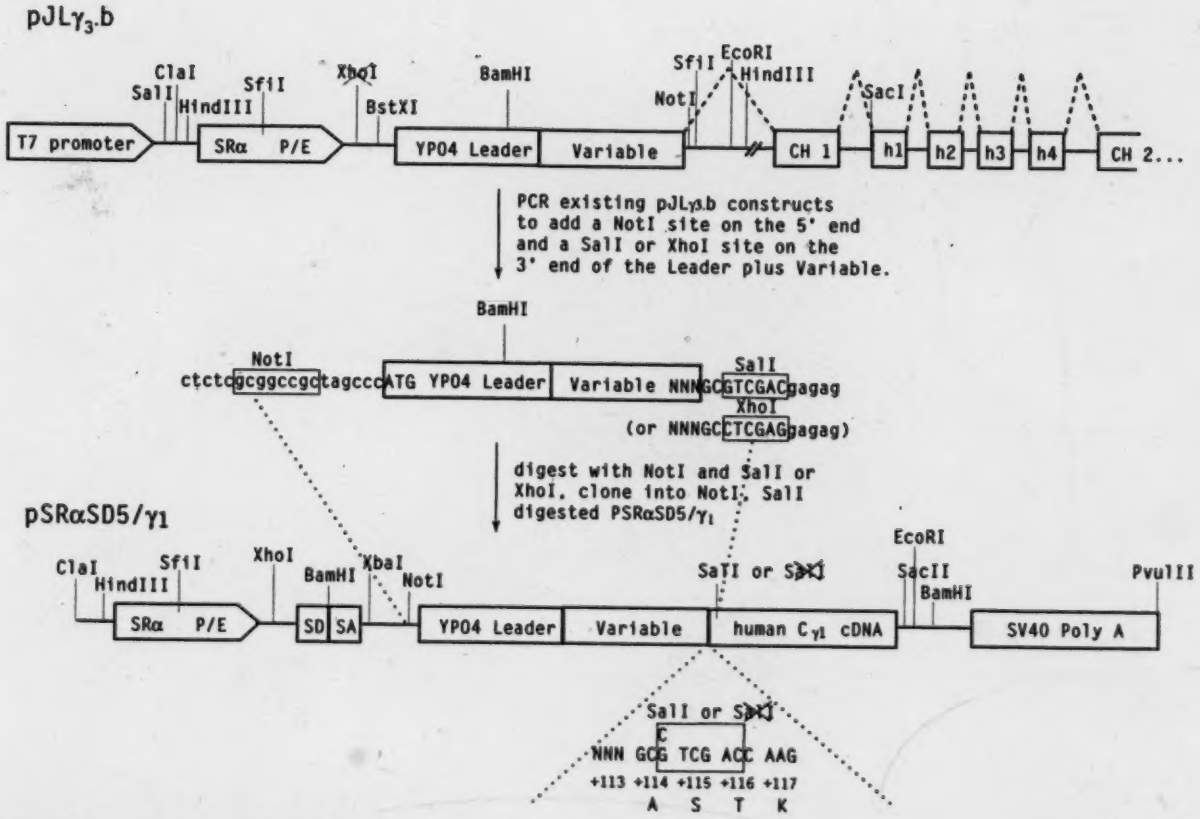


Figure 46. Strategy for construction of heavy chain $\gamma_1$constructs in SRαSD5/γ_1. The Kozak consensus sequence was omited from the 5' *Not*I primers used for these constructions.

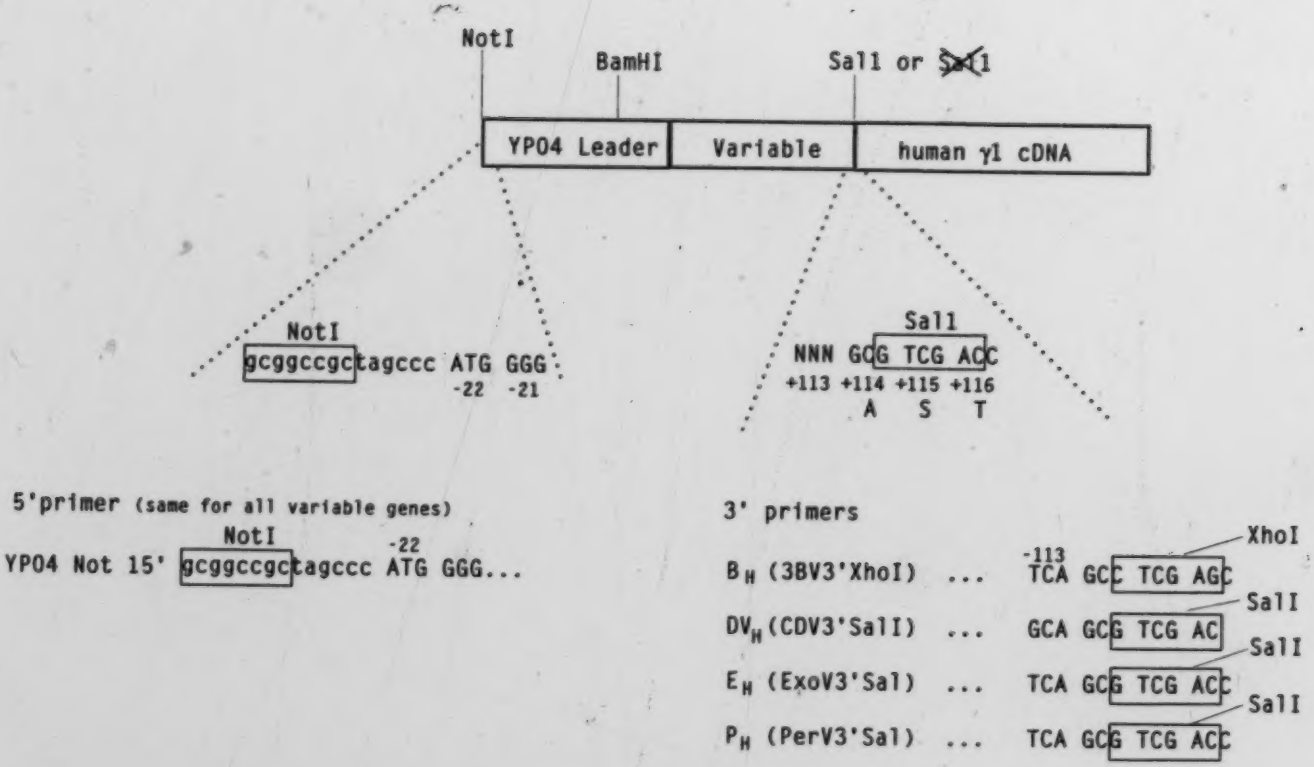


Figure 47. Details of the primers used to create the various V_H /Cγ1 constructs.

methionine present in the original YP04 leader sequence (amino acid -24, figure 40), so that the second methionine in YP04 (residue -22) became the initiating codon. A unique 3' primer containing either a *Sal*I or *Xho*I restriction site was designed for each V_H, whereas the 5' primer containing the NotI site was the same for all of the PCR reactions (figure 47 and table 4.

H. *Expression of B [PK3B], E [Ex0-s], and P [18B-13-41] IgG1 chimeric antibodies*

To obtain chimeric IgG1 immunoglobulins for antibodies B, E, and P, γ1.c constructs containing each of the various V_H segments were co-transfected into COS cells along with the corresponding V_L segments in the k.b expression vector. To obtain the chimeric IgG1 for antibody D, the same γ1.c vector was used to produce the heavy chain, but a slightly different human kappa chain expression vector, called version k.c was used.

I. *Construction of version k.c expression vector; expression of the D [CD4-TT] IgG1 chimeric antibody*

The 'version c' kappa expression plasmid was derived from the γ1.c vector as shown in figure 48. Essentially the human γ1 cDNA is replaced with a PCR amplified human kappa coding element, and the YP04 leader plus V_L (also generated by PCR) is fused in frame to the kappa element. Because of the presence of a *Xho*I site at the point of fusion, two amino acids are altered from the original C kappa protein (figure 48). Motivation for the construction of the k.c vector was two-fold: 1) we wished to create and test an 'intronless' human kappa expression vector, which would be strictly analogous with the γ1.c version vector, and 2) once it existed, such a k.c vector would make it very simple to conduct 'variable region swapping' as described in chapter 5. This construction was done initially only for V_L of the D antibody. Thus to obtain the chimeric IgG1 for antibody D, the γ1.c vector containing the V_H from antibody D (hereafter abbreviated pD(V_H)γ1.c) was co-transfected along with the k.c vector containing the V_L from antibody D (abbreviated pD(V_L)k.c).

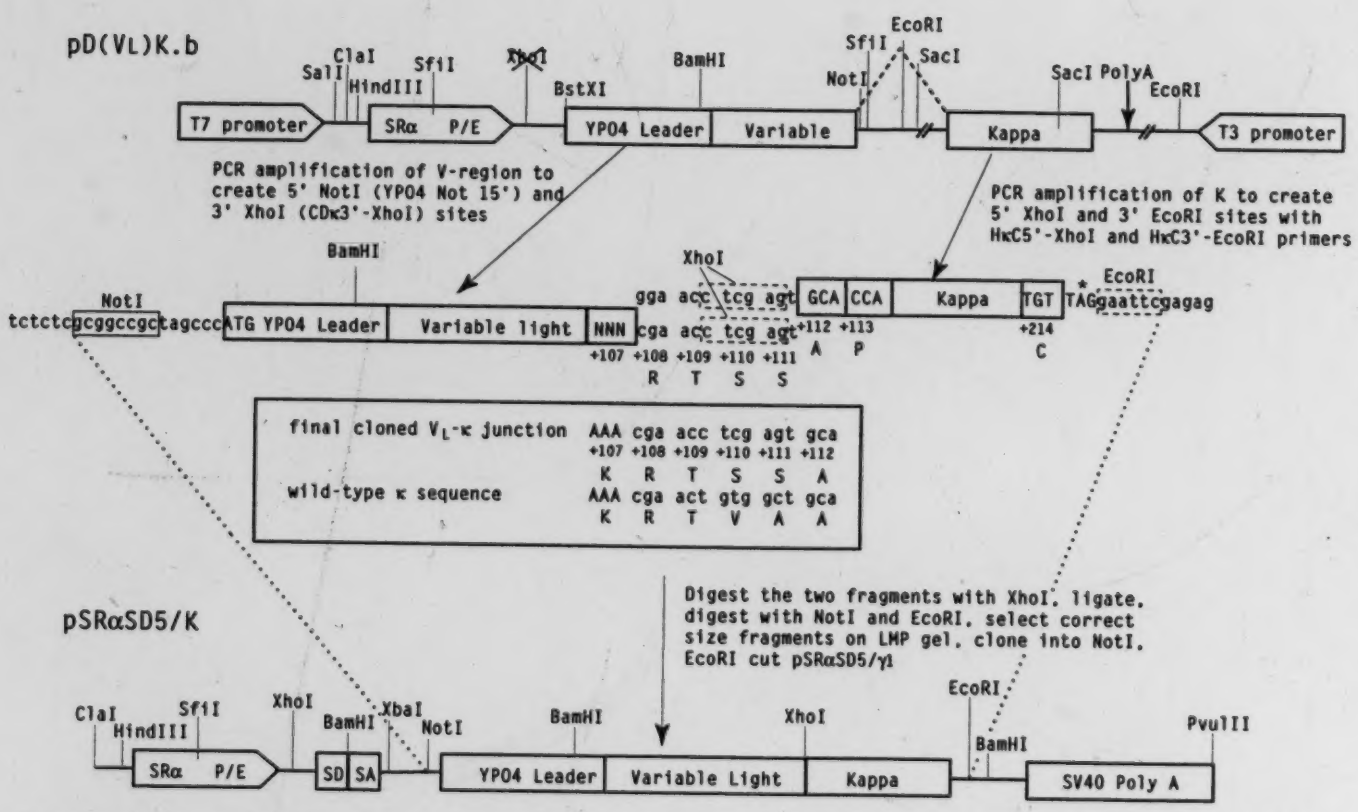


Figure 48. 'Version K.c' expression vector. Strategy for cloning VL-Kappa chain constructs without internal introns.

J. *Analysis of secreted chimeric mouse/human IgG3 and IgG1 antibodies by western blots*

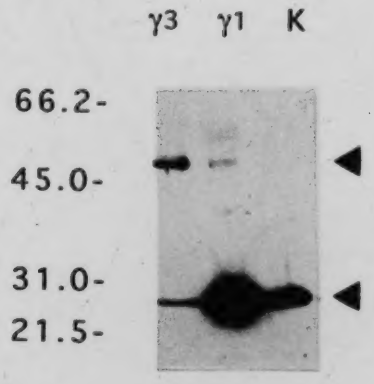
As described above, COS cells were transiently transfected with either γ3.b or γ1.c plasmids containing B, D, E, or P VHs in combination with the appropriate VLs in either the k.b or k.c plasmids. The transfected COS cells were cultured for 72-96 hours and then supernatants were collected and concentrated 5 to 10 fold by dialysis against solid PEG-(chapter 2). The concentrated supernatants were initially screened for the presence of chimeric antibodies by sandwich ELISA (detects human IgG H and L constant regions; data not shown). Supernatants giving positive results were further analyzed by separation on both reducing and non-reducing SDS-PAGE gels and Western blotting, this time

probing with a conjugated goat anti-human IgG[H+L] antisera. Use of the reducing gels ensured that the expressed chimeric chains exhibited the appropriate molecular weights (about 25 kD for the light chain and 51-56 kD for the heavy chain), and thus contained both C and V regions. Use of the non-reducing gels ensured that the H and L chains were properly associated via disulfide bonds.

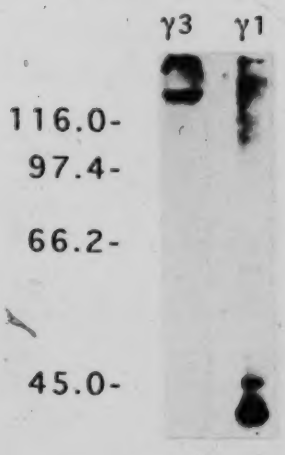
Results of the western blots analyzing the various chimeric antibodies are shown in figures 49 and 50. In all cases western blots made from reducing gels showed that H and L chains of the expected sizes were present in the COS supernatants. In most cases the intensity of the heavy and light chain bands were approximately equal, although this is not true in every case (see below). When the same supernatants were run on non-reducing gels and western blotted, the light chain and heavy chain monomer bands disappeared, and these were replaced in all cases by one or a few high molecular weight bands (>97 kD), indicating that the H and L chains were associating as expected. In addition to the high molecular weight bands, figures 50 B and 51 E and P show bands at ≈20kD and/or ≈45kD, which are most likely light chain monomers or dimers. Presence of these smaller molecular weight bands in the non-reducing gels suggests that in some

FIGURE 49. Western blots of chimeric B (B) and D (D) proteins in a reduced (red.) and non-reduced (non-red.) state, with sizes of protein markers expressed in kD. γ3 is a chimeric IgG3 (i.e. cells transfected with X(V_H)γ.b + X(V_L)K.b, where X is either B or D). γ1 is a chimeric IgG1 (i.e. cells transfected with X(V_H)γ.c +B(V_L)K.b/D(V_L)K.c, where X is either B or D). K is a chimeric kappa chain alone (i.e. cells transfected with B(V_L)K.b).

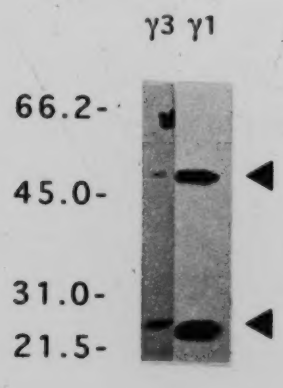
B red.



B non-red.



D red.



D non-red.

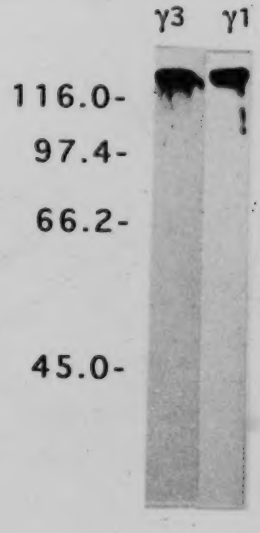
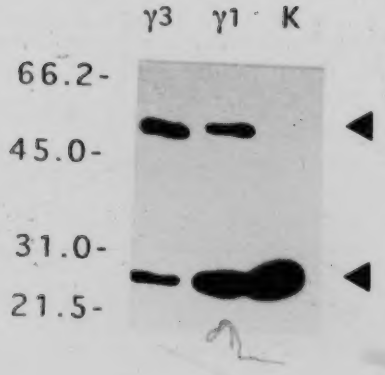
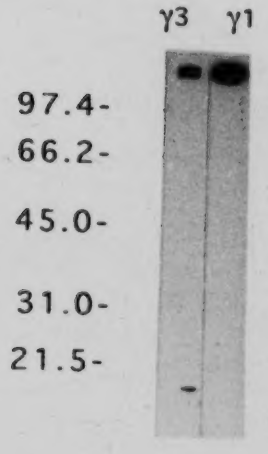


FIGURE 50. Western blots of chimeric E (E) and P (P) proteins in a reduced (red.) and non-reduced (non-red.) state, with sizes of protein markers expressed in kD. γ3 is a chimeric IgG3 (i.e. cells transfected with X(V_H)γ.b + X(V_L)K.b, where X is either E or P). γ1 is a chimeric IgG1 (i.e. cells transfected with X(V_H)γ.c + X(V_L)K.b, where X is either E or P). K is a chimeric kappa chain alone (i.e. cells transfected with E(V_L)K.b). The light chains should be of the same size, except for some curvature in the gel.

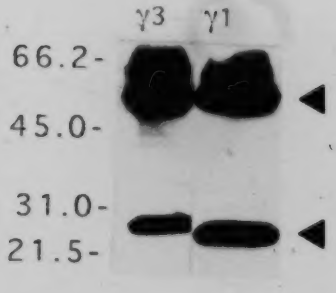
E red.



E non-red.



P red.



P non-red.

cases there is an imbalance between production of H and L chains, and/or that formation of H and L heterodimers is somewhat unfavorable.

K. *Analysis of secreted chimeric mouse/human IgG3 and IgG1 antibodies by competitive ELISA*

All four of the original mouse monoclonal antibodies used to generate chimeric constructs were capable of binding to pili of the K strain of *Pseudomonas aeruginosa* (also known as 'PAK' pili). An important question was whether or not the binding of our chimeric antibodies to PAK pili was comparable to that of the parental mouse antibodies. To obtain a relative measure of antibody binding affinity for both the original mouse as well as the new chimeric antibodies we used a competitive ELISA assay. Ideally this data would have been obtained by equilibrium dialysis; however the competitive ELISA assay provides a simple and more rapid approach, and the assay was readily available to us through Dr. R. Irvin's laboratory.

The first step in the competitive assay involved coating a microtitre plate with affinity purified goat anti-human (or anti-mouse) IgG-Fc antibodies, blocking, and then incubation of concentrated COS cell supernatants to capture the chimeric antibodies onto the plate. The plates were washed, blocked again, and then incubated with a fixed concentration of biotinylated PAK pili (1.7 μg/ml) pre-mixed with various known concentrations of non-biotinylated PAK pili. Plates were washed again and the quantity of biotinylated pili bound to the captured chimeric (or wild-type mouse) antibody was measured using a streptavidin conjugated enzyme and chromogenic substrate. For each concentration of non-biotinylated pili (e.g. competitor) assayed, the optical absorption value obtained was converted to percent inhibition of binding, using the formula [1-(absorption in the presence of competitor/absorption in the absence of competitor)] x 100. Percent inhibition of binding was then plotted versus log concentration of competitor, as shown for example in figure 51, where the chimeric B-IgG3, B-IgG1, and B-light chain antibodies were tested. In general the percentage inhibition increases

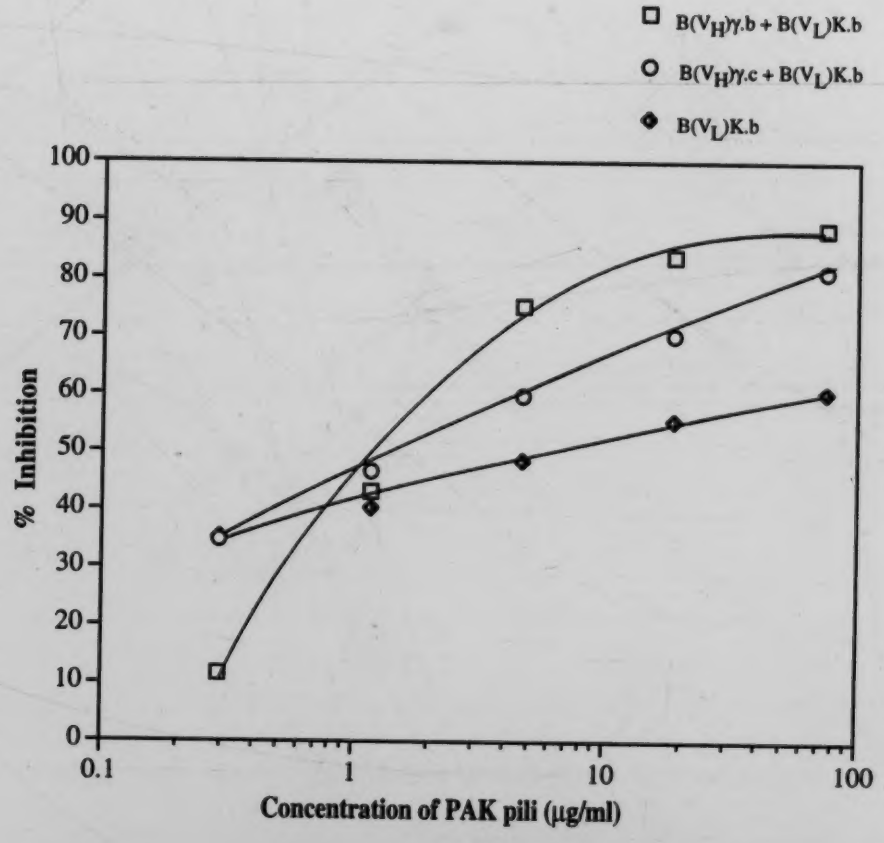


Figure 51. Representative competitive ELISA profiles of B chimeric constructs showing the inhibitory effect of adding non biotinilated PAK pili on the binding of B constructs to biotinilated PAK pili. Microtitre plates were coated with anti-human (or anti-mouse) IgG-Fc antibodies, this was followed by blocking, and then incubation of concentrated COS cell supernatants to capture the chimeric antibodies onto the plate. The plates were washed, blocked again, and then incubated with a fixed concentration of biotinilated PAK pili (1.7 μg/ml) pre-mixed with various known concentrations of non-biotinylated PAK pili. Plates were washed again and the quantity of biotinilated pili bound to the captured chimeric (or wild-type mouse) antibody was measured using a streptavidin conjugated enzyme and chromogenic substrate. For each concentration of non-biotinilated pili (e.g. competitor) assayed, the optical absorption value obtained was converted to percent inhibition of binding, using the formula [1-(absorption in the presence of competitor/absorption in the absence of competitor)] x 100. Percent inhibition of binding was then plotted versus log concentration of competitor. In general the percentage inhibition increases as the concentration of competitor is increased. The concentration of inhibitor (in μg/ml) required to cause 50% inhibition of binding is called the I50, and this gives a relative measure of the affinity of the antibody for PAK pili. See table 6 and 7 for tabulated I50 for all the developed constructs.

as the concentration of competitor is increased. For an antibody that binds strongly (e.g. B-IgG3, figure 51), a relatively low concentration of competitor (1.2 μg/ml) is required to inhibit 50% of the binding of the biotinylated pili, whereas for a more weakly binding antibody a much higher concentration of competitor is required to achieve the same inhibitory effect.

The concentration of inhibitor (in μg/ml) required to cause 50% inhibition of binding is called the I_{50}, and this gives a relative measure of the affinity of the antibody for the PAK pili. In addition, Nieto (322) has defined the 'apparent association constant' (aKa) as the reciprocal of the I_{50}, which has the advantage that the value increases as the antibody affinity increases. Table 6 summarizes the I_{50} and aKa values obtained for the original mouse antibodies B, D, P, and E (from ascites; analyzed by western blotting as shown in figure 52) as well as for the mouse/human chimeric IgG3 and IgG1 antibodies produced from transfected COS cells. These values were obtained from the a whole series of competitive ELISA experiments, the binding curves for which are included in Appendix 1 at the back of this thesis.

The original mouse monoclonal antibodies had aKa values ranging from ≈170 to 20 ml/g, and could be ordered B≈E>D>P (highest to lowest affinity). Surprisingly the IgG3 chimeras showed higher aKa values for all antibodies (ranging from ≈830 to 140 ml/g) and retained nearly the same order (B≈E>D≈P). However, except for B, the IgG1 chimeras did not perform as well as the IgG3 chimeras (with aKa values ranging from ≈625 to 20 ml/g). This is still as good or better than the original mouse antibodies, except for the chimeric IgG1-E which appears to have lost most of it's binding (ordering B>>D=P≥E).

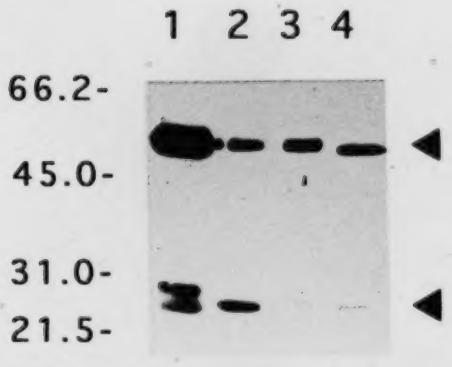
TABLE 6. *The amount of non-biotinilated PAK pili needed to cause 50% Inhibition in binding of mouse , chimeric IgG3 and IgG1 antibodies to biotinilated PAK pili* (I_{50}) and the corresponding apparent association constant (aKa) *

Mouse Antibody	I_{50} @	aKa	Binding class
B	5.8 ± 2.4	172.4	M
D	16 ± 16.7	62.5	L
E #	6.7	149.3	M
P	51.7 ± 44.3	19.3	L
Chimeric mouse/human IgG3 antibody			
$B(V_H)\gamma.b$ + $B(V_L)k.b$	1.2±0.5	833.3	H
$D(V_H)\gamma.b$ + $D(V_L)k.b$	7.2±2.8	138.9	M
$E(V_H)\gamma.b$ + $E(V_L)k.b$	2.0±0.8	500	H
$P(V_H)\gamma.b$ + $P(V_L)k.b$	5.6±5.5	178.6	M
Chimeric mouse/human IgG1 antibody			
$B(V_H)\gamma.c$ + $B(V_L)k.b$	1.6±0.4	625	H
$D(V_H)\gamma.c$ + $D(V_L)k.c$	21.2±10.2	47.2	L
$E(V_H)\gamma.c$ + $E(V_L)k.b$	47.3±15.0	21.1	L
$P(V_H)\gamma.c$ + $P(V_L)k.b$	25.3±10.6	39.5	L

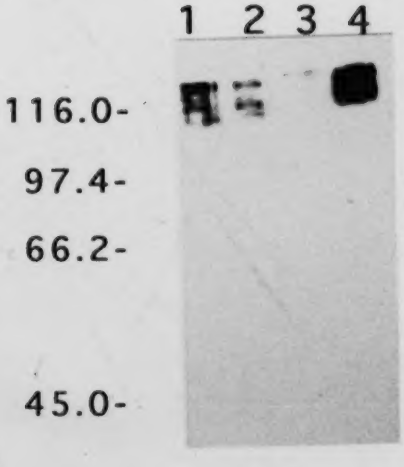
* Each experiment was repeated at least three times and the average was tabulated. # E value is the result of only one experiment. @ I_{50} is expressed in µg/ml ± standard deviation (see Appendix 1). aKa is expressed in ml/g (due to the heterogeniety in the size of PAK pili) and it = $1/I_{50}$. A classification of functional binding affinities [high (H), moderate (M) and low (L) categories] was based on the work of Doig *et al.* (105), who found that the mouse monoclonal antibody PK99H, which had an aKa of 300 ml/g, could effectively block *Pseudomonas aeruginosa* strain PAK from binding to BECs, as well as passively protect mice from lethal injections with Pseudomonas. Thus I classified them as high (aKa ≥ 300 ml/g; e.g. aKa ≥ that of PK99H), moderate (aKa 100-300 ml/g, down to 1/3 of PK99H) and low (aKa <100 ml/g)..

Figure 52. Western blots of the original Mouse antibodies. **A** represents the reduced and **B** the non-reduced state with sizes of protein markers expressed in kD. Lane **1** is P, lane **2** is E, lane **3** is D, and lane **4** is B.

A



B



CHAPTER V

CHARACTERIZATION OF NOVEL CHIMERIC CONSTRUCTS--HEAVY OR LIGHT CHAINS ALONE, LIGHT CHAIN EXCHANGE, AND VARIABLE REGION SWAPPING

A. *Introduction*

Once the DNA constructs encoding chimeric antibodies are made, it becomes possible to perform 'mixing and matching' experiments, where elements can be combined in ways which do not occur in nature. In this chapter, three different kinds of experiments were performed: 1) heavy or light chain chimeric constructs alone were transfected into COS cells, and if the supernatants contained recombinant proteins (as assayed by western blotting), these were tested by competitive ELISA to determine the relative contributions of the V_H and the V_L to overall binding to PAK pili, 2) the Pγ1 chimeric heavy chain was combined with the B, D, and E light chain chimeric constructs, in order to try and improve the affinity of the Pγ1 molecule, and 3) the D antibody V_H was fused to the human Ck, and the D-V_L fused to the human Cγ1. Motivation for this last set of experiments arose because the D-V_H γ3 chain alone was secreted and had a high affinity for PAK pili, and we were interested to know if D-V_H attached to Ck alone would provide a more 'minimal' antibody with the same high affinity, and/or if two D-V_Hs (e.g. one attached to Cγ1 and one to Ck) would have an even higher affinity.

Results of western blotting and competitive ELISA experiments suggested that the 'light chain exchange' experiments were quite successful, since all combinations of heavy and light chains were assembled and secreted, and the resulting antibodies showed higher aKa values than the original P [18B-13-41] IgG1 chimeric antibody. To perform the 'variable region swapping' experiments two more constructs were required,

based on the γ1.c and k.c vectors described in Chapter 4. Whereas western blotting indicated that the 'swapped' proteins were assembled and secreted, the competitive ELISA results obtained using these proteins were more ambiguous.

B. *Characterization of singly transfected chimeric heavy chains or light chains by western blotting and competitive ELISA*

I transfected COS cells with individual plasmids corresponding to each of the various heavy or light chain constructs described in Chapter 4. The supernatants were concentrated as before and analyzed on reducing and non-reducing western blots. For all four antibodies the light chain chimeras were secreted, and in the case of P and D clearly formed dimers (see figure 49 for antibody B, figure 53 for D, figure 50 for E, and 54 for P). Surprisingly, two of the heavy chain γ3 constructs (D and P) were also secreted as single chains, and both of these appeared to form inter-chain disulfide bonds, since they ran as higher molecular weight bands in the unreduced gels (figures 53 and 54). All other heavy chains (e.g. γ3 forms of B and E; γ1 forms of B, D, E, P) were not secreted when expressed in isolation (data not shown).

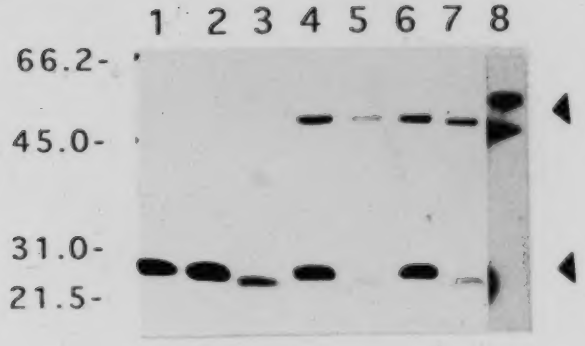
Results for the competitive ELISA experiments using the secreted single heavy or light chains are shown in table 7. Some of the single chains (notably the D and P γ3 heavy chains, and the B light chain) had aKa values suggesting moderate to high affinity for the antigen. The D light chain is less certain, with the 'version b' construct showing moderate affinity, but with a high standard deviation, whereas the 'version c' construct appears to be of lower affinity, with more reliable data.

C. *Western blotting and competitive ELISA analysis of the Pγ1 [18B-13-41] heavy chain expressed with various chimeric light chains*

In this series of experiments COS cells were transfected with the plasmid encoding the Pγ1 chimeric construct (e.g. pP(V_H)γ1.c) together with one of the following plasmids: pB(V_L)k.b, pD(V_L)k.b, or pE(V_L)k.b. All three combinations appeared to result in secreted H and L chains which assembled to form high molecular weight bands

FIGURE 53. Western blots of chimeric D proteins in a reduced (red.) and non-reduced (non-red.) state, with sizes of protein markers expressed in kD. Lane **1**, D(VL)K.b transfection, lane **2**, D(VL)K.c transfection, lane **3**, D(VH)K.c transfection, lane **4**, D(VL)γ.c + D(VL)K.c transfection, lane **5**, D(VL)γ.c + D(VH)K.c transfection, lane **6**, D(VL)γ.c + D(VL)K.b transfection, lane **7**, D(VH)γ.c + D(VH)K.c transfection, and lane **8**, D(VH)γ.b transfection.

D red.



D non-red.

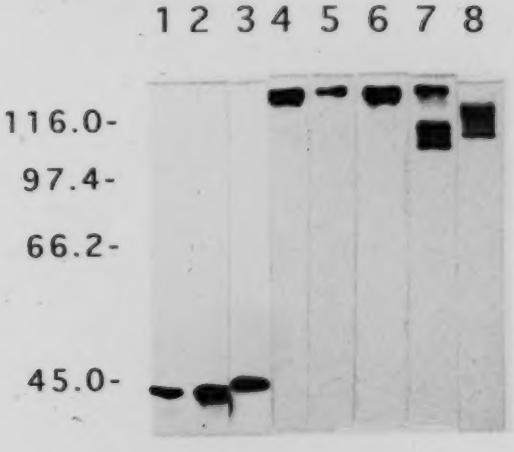
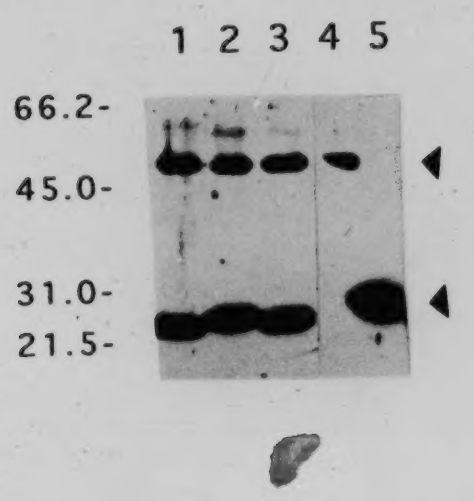


FIGURE 54. Western blots of chimeric P proteins in a reduced (red.) and non-reduced (non-red.) state, with sizes of protein markers expressed in kD. Lane **1**, P(VH)γ.c + E(VL)K.b transfection, lane **2**, P(VH)γ.c + D(VL)K.b transfection, lane **3**, P(VH)γ.c + B(VL)K.b transfection, lane **4**, P(VH)γ.b transfection, and lane **5**, P(VL)K.b transfection.

Red.



Non-red.

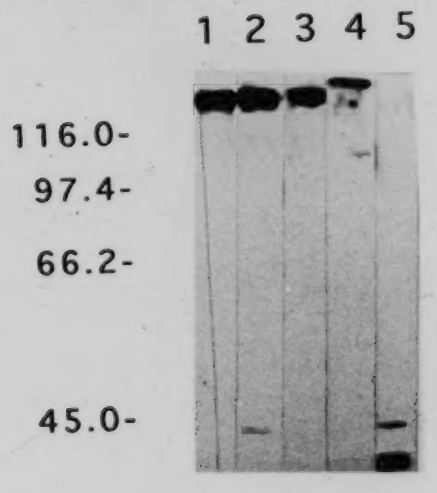


TABLE 7. *The amount of non-biotiniated PAK pili needed to cause 50% Inhibition in the binding of the different chimeric constructs to biotiniated PAK pili.* (I50) and the corresponding apparent association constant (aKa) *

Chimeric constructs: Heavy Light	I50	aKa	Binding class
a- Heavy chain alone:			
D(V_H)γ.b	3.0 ± 1.0	333	H
P(V_H)γ.b	8.8 ± 4.8	113.6	M
b- Light chain alone:			
B(V_L)k.b	7.6 ± 3.0	131.6	M
D(V_L)k.b	9.1 ± 7.7	109.9	M
D(V_L)k.c	26.7 ± 7.6	37.5	L
E(V_L)k.b	49.7 ± 1.5	20.1	L
P(V_L)k.b	21.5 ± 15.3	46.5	L
c- Chimeric mouse/human IgG3 antibody			
B(V_H)γ.b + B(V_L)k.b	1.2 ± 0.5	833.3	H
D(V_H)γ.b + D(V_L)k.b	7.2 ± 2.8	138.9	M
E(V_H)γ.b + E(V_L)k.b	2.0 ± 0.8	500	H
P(V_H)γ.b + P(V_L)k.b	5.6 ± 5.5	178.6	M
d- Chimeric mouse/human IgG1 antibody			
B(V_H)γ.c + B(V_L)k.b	1.6 ± 0.4	625	H
D(V_H)γ.c + D(V_L)k.c	21.2 ± 10.2	47.2	L
E(V_H)γ.c + E(V_L)k.b	47.3 ± 15.0	21.1	L
P(V_H)γ.c + P(V_L)k.b	25.3 ± 10.6	39.5	L
e- P antibody Light-chain exchange			
P(V_H)γ.c + B(V_L)k.b	2.1 ± 0.3	476.2	H
P(V_H)γ.c + D(V_L)k.b	5.0 ± 3.2	200	M
P(V_H)γ.c + E(V_L)k.b	7.1 ± 6.1	140.8	M
f- D antibody variable-region swapping			
D(V_H)k.c	45.3±12.7	22.1	L
D(V_H)γ.c + D(V_H)k.c	18.7 #	53.5	L
D(V_L)γ.c + D(V_L)k.b	30 ± 12.5	33.3	L
D(V_L)γ.c + D(V_L)k.c	11.2 ± 3.3	89.3	L
D(V_L)γ.c + D(V_H)k.c	15.6 ± 10.6	64.1	L

Unlike the other values, this number is the average of only two experiments, all the othes are averages of at least three experiments. * I50 is expressed in µg/ml ± standard deviation (see Fig. 51 and Appendix 1). aKa isexpressed in ml/g (due to the heterogeniety in the size of PAK pili) and it = 1/I50. A classification of functional binding affinities is described in table 6. The c and d parts are from table 6.

on non-reducing gels, as shown in figure 54. Supernatants from the pD(VL)k.b transfection also showed a small quantity of light chain dimers (lane 2, figure 54).

Competitive ELISA results for these 'light chain exchange' experiments are shown in table 7. In comparison to the original P IgG1 antibody (which contained the P light chain), presence of the B, D, or E light chains all appeared to increase the affinity of the resulting antibody molecule. The combination containing the B light chain had the highest affinity (12 fold better than the original P IgG1), with the D and E light chains yielding antibodies of somewhat lower, but still improved affinity.

D. *Construction of expression vectors where VH is fused to Ck and VL is fused to Cγ1 [CD4-TT]*

Details of the cloning strategy used to construct these expression vectors are given in figures 55 and 56. These constructions are closely related, and can be compared to the 'version γ1.c' and 'version k.c' vectors described in chapter 4, figures 45 and 48. The choice of amino acids at the fusion points (e.g. where VH joins kappa, and VL joins γ1) was purely practical, based on the restriction sites being used. In the VL to γ1 construct (figure 55; called pD(VL)γ1.c), the first two residues of the kappa constant region (residues 108 and 109; R T) are joined to the second residue of the γ1 constant region (residue 115; S), without any amino acid changes to either constant region. In contrast, in the VH to k construct (figure 56; called pD(VH)k.c), the first residue of the γ1 constant region (residue 114; A) is joined to the second residue of the kappa constant region (residue 109), but residues 109, 110, and 111 of the kappa constant region are altered from TVA to ASS in order to accommodate the *XhoI/ Sal* I connection (figure 56).

E. *Western blotting and competitive ELISA analysis of the D [CD4-TT] antibody molecules where VH and VL regions were swapped*

In this series of experiments COS cells were transfected with the plasmids pD(VL)γ1.c and pD(VH)k.c either singly, or in combination with each other, or in

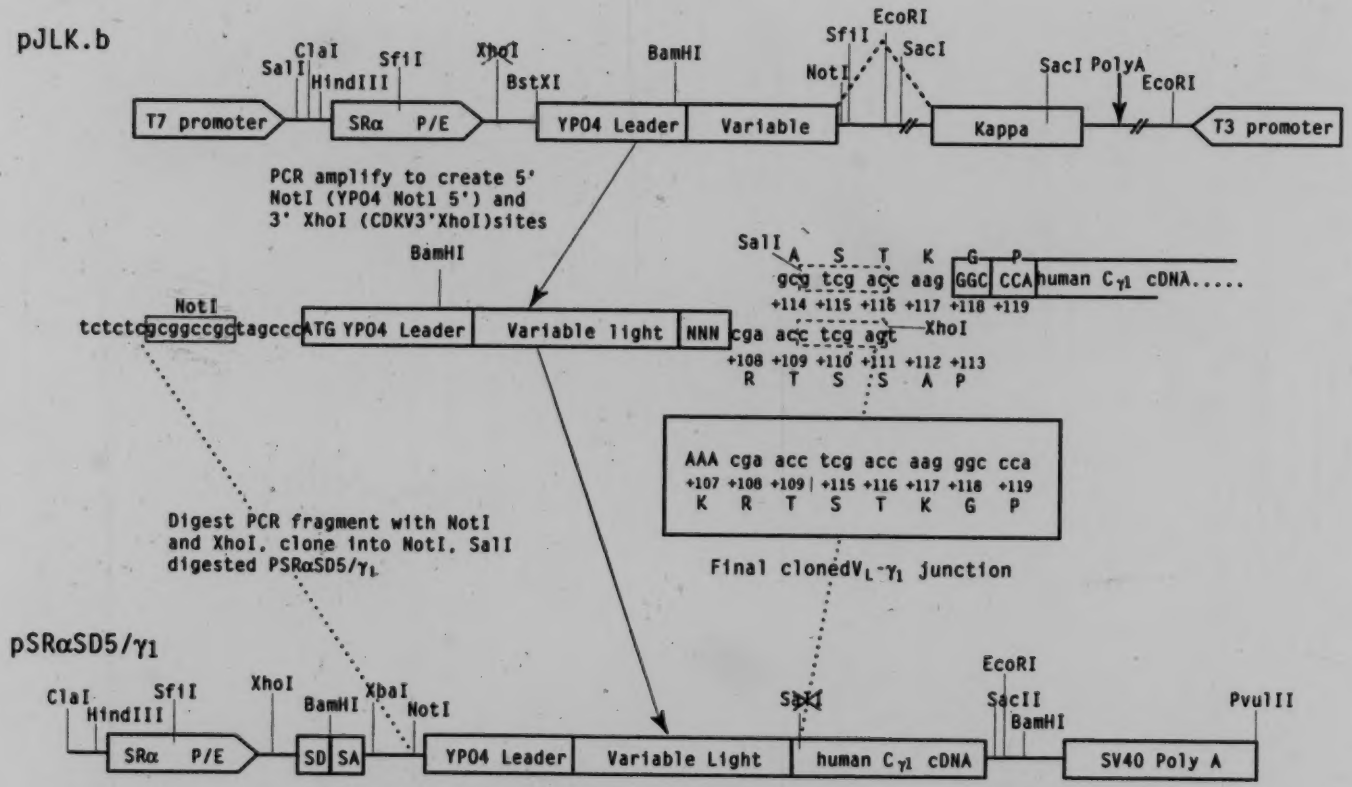


Figure 55. Strategy for cloning light chain variable gene constructs fused to human Cγ1 (VL/Cγ1) coding element, showing details of the fusion of the VL with the Cγ1. Codons are numbered according to the kappa chain for VL, and according to Cγ1 for the constant region segment.

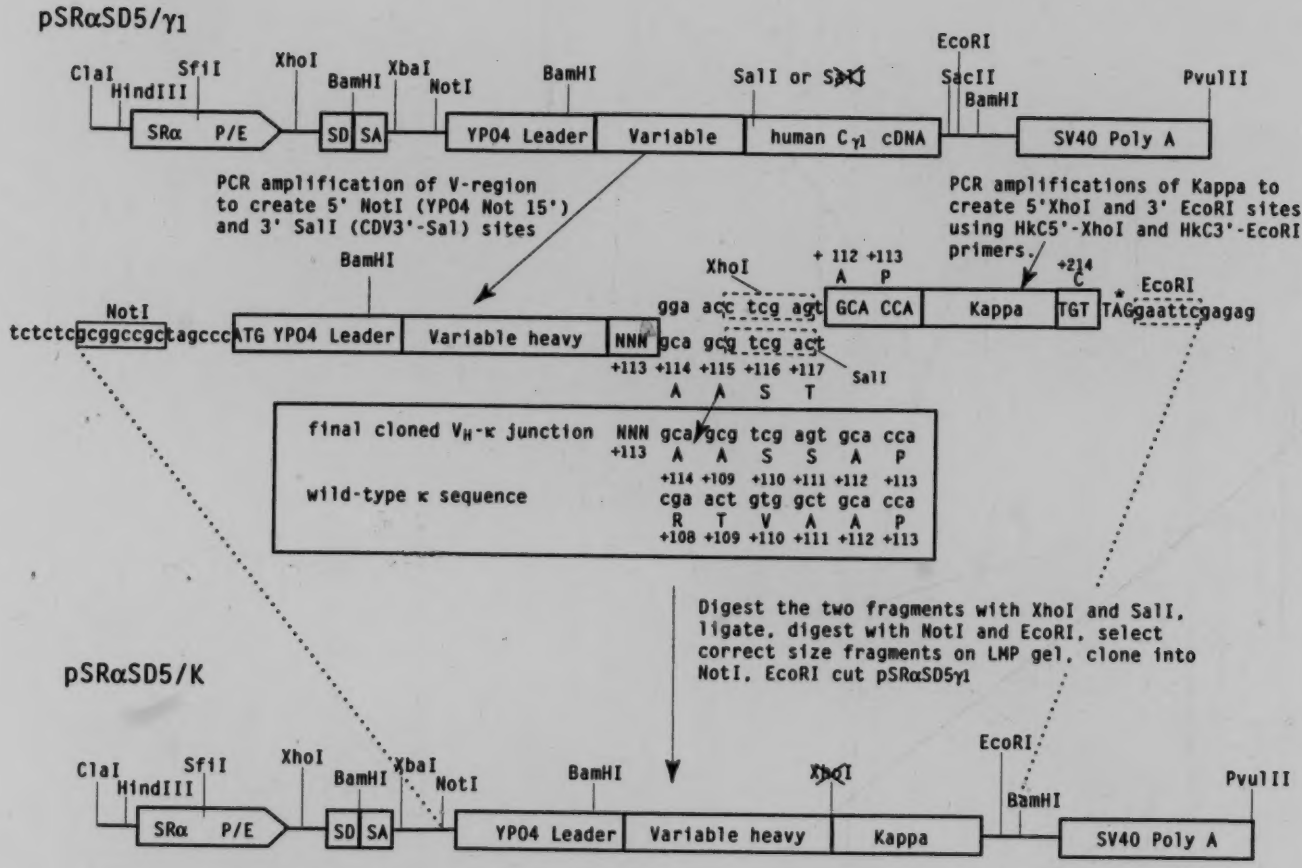


Figure 56. Strategy for cloning heavy chain variable gene constructs fused to the kappa coding element.

combination with plasmids pD(V_H)γ1.c, pD(V_L)k.b, or pD(V_L)k.c (see table 7, which lists the various transfections). As with the other γ1 constructs, when transfected by itself pD(V_L)γ1.c did not result in any secreted product (data not shown), whereas pD(V_H)k.c resulted in secreted light chains which formed dimers (figure 53). In the other four transfections where combinations of plasmids containing Cγ1 and Ck were used, the expected heavy and light chains were secreted into the supernatant (figure 53). Furthermore, western blots made from non-reducing gels showed high molecular weight bands (figure 53; bands >116 kD), suggesting that the H and L chains were assembling via interchain disulfide bonds. Interestingly, the pD(V_H)γ1.c plus pD(V_H)k.c transfection showed an additional band or bands at ≈100 kD, which could represent a heavy-chain dimer (figure 53, lane 7).

All of the supernatants which contained chimeric antibodies by western blotting were analyzed by competitive ELISA, and these results are summarized in table 7. All of the resulting antibodies had roughly the same affinity as the chimeric D IgG1 (and the original mouse D monoclonal), suggesting that although the novel antibodies were working to some degree, the advantage gained by V-region swapping was not nearly as dramatic as was seen for example with the light chain exchange experiment described above. Surprisingly none of these novel constructs had as high an affinity as the D γ3 chimeric chain secreted by itself.

CHAPTER VI
DISCUSSION AND CONCLUSIONS

A. *Introduction and summary of results*

The primary goal of this project was to chimerize five mouse MAbs with specificity for *Pseudomonas aeruginosa* pili. Four of these monoclonals (PK99H, E, D, and P) protected mice against a lethal dose of *Pseudomonas aeruginosa.* One monoclonal (B) did not, however, it was carried along to be used as a negative control. The objective was to produce at least one chimeric antibody with high affinity for pili, and with the potential of providing protection against *Pseudomonas aeruginosa* infections in human clinical trials. The objective was met by producing such potentially therapeutic molecules (results presented in chapter 4). In addition, information obtained from this work is currently being used by a commercial laboratory for the next phase involving the construction of humanized forms (and fragments thereof that retain antigen binding capability). Such constructs are to be used as prophylactics for the prevention of *Pseudomonas* infections in human clinical trials.

The first step in the chimerization process involved the isolation of cDNA that coded for the heavy and light chain variable regions. This was achieved by the construction and screening of cDNA libraries (in the case of PK99H and B) and the use of the inverted polymerase chain reaction (for D, E, and P). V-region sequences representing the heavy and light chain of four of the five studied antibodies were presented in chapter 3. In this chapter I will discuss the efficiency of the two methods used and analyze some of the unexpected results. I will also discuss the uniqueness of the isolated sequences, and what information can be obtained that can aid in the next phase of humanization .

To produce the chimeric molecules, the isolated V-region sequences were then cloned into expression vectors containing the γ3, K, or γ1 human constant regions. The V-region containing vectors were later co-transfected (γ3 + K, or γ1 + K) into COS cells with the resulting assembly and secretion of the IgG3 and the IgG1 chimeric forms. Steps involved in vector construction were presented in chapter 4, as well as western data verifying the assembly and secretion of these molecules. In this chapter I will discuss the unexpected results obtained from constructing the different vector versions and analyze the data obtained from the western experiments.

To test the antigen binding affinities of the IgG3 and the IgG1 chimeric forms in comparison to the mouse MAbs, a competitive ELISA was performed. Affinity data expressed as I_{50} and the corresponding aKas were presented in chapter 4. The chimeric antibodies of the IgG3 form had affinities higher than the original mouse antibodies, verifying the conclusion that the goal was met in the production of chimeric molecules with a human therapeutic potential. In this chapter I will discuss the binding affinities of the different chimeric forms and its relevance to protection against *Pseudomonas* infections.

Novel constructs were also made by the use of the 'light-chain exchange' and the 'variable-region swapping' approaches. Transfection, secretion and binding of these novel constructs (as well as that of the 'heavy and light chain alone' transfections) were presented in chapter 5. These results confirmed the rare secretion (and antigen binding) of solitary heavy chains, and the validity of the 'light chain exchange' as a possible approach for increasing antigen binding affinity. In this chapter , I will discuss the relevance of these novel approaches, the successes and the unexpected results.

B. *Isolation of V-region cDNAs*

Failure to obtain V_H and V_L cDNAs from the PK99H hybridoma- Overall I was successful in obtaining the cDNA sequences for four of the five original antibodies which we set out to study. The one antibody which I was unable to obtain cDNA

sequences for was PK99H. We first attempted to clone functional H and L chain cDNAs by screening cDNA libraries. This turned out to be simple and straightforward (albeit time consuming) for the B antibody, whereas it proved to be impossible for the PK99H antibody. It appears likely that although the original PK99H hybridoma produced sufficient H and L chains to enable N-terminal protein sequencing (e.g. the protein sequence is shown in figure 13), by the time the same cell line was used to generate RNA for this cloning experiment, it had lost protein expression, and consequently there was little or no specific H and L chain mRNA present in the harvested nucleic acids. In addition to the failure to obtain the desired clones, this idea is supported by three other observations: 1) as described early in chapter 3, the signals on northern blots made from PK99H RNA and probed with mouse Cγ1 or Ck probes were very weak (especially the Cγ1) in comparison to the signals seen in similar northerns made from the B hybridoma RNA, 2) the frequency of Cγ1 positive clones (1 per 66,000) was much lower in the PK99H cDNA library than in the B library (1.3 per 1000), even considering the fact that the B library was made from poly A+ RNA, which would be expected to increase frequency of heavy chain mRNAs by at most 10-20 fold, and 3) instead of isolating a 'well behaved' cDNA encoding the PK99H light chain, all kappa positive clones encoded an aberrant light chain known to be produced by the NS-1 myeloma fusion partner (317) (figure 22). One possible reason for the loss of Ig expression by the PK99H hybridoma cells is the loss of the corresponding segment of DNA containing the rearranged Ig genes, however proof for this idea would require more extensive molecular-genetic analysis.

It has been reported that in general hybridoma cell lines generate immunoglobulin secretion-loss mutants (which usually outgrow the Ig secreting parent) at a frequency of one in 10^{-2} to 10^{-3} cells, and in some cell lines this frequency can be as high as 10% (283). This emphasizes the need to continually re-clone hybridoma cell lines and select for high-producer cell lines, which probably was not done to the PK99H cell line prior

to our obtaining the cells for production of RNA. The simple conclusion here is that in order to isolate the desired cDNA, in all cases single-cell cloning and selection for a high-antibody producing hybridoma should be done just prior to harvesting of the RNA.

Although we were unable to isolate a cDNA encoding the mature PK99H heavy chain, we did isolate three clones which cross-reacted with the PK99H VH specific probe (probe 8a5, generated by PCR based on the N-terminal protein sequence; figure 15). Each of the clones isolated contained a unique sequence, and although one of them even cross-hybridized with the Cγ1 sequence, all three sequences contained either heptamer/nanomer recombination signals and/or introns , and therefore did not represent mature mRNAs. Examples of intron-containing cDNAs have been reported (163). As to the origin of these transcripts, since every VH segment has a promoter in the 5' flanking region (see ref. (323) for a review), these clones could represent run-off transcripts from germline (e.g. unrearranged) genes. Alternately they could also be transcripts from non-functionally rearranged genes, since it has been reported that in hybridomas such rearranged genes can be transcriptionally active (324). Since in the case of PK99H no further selection for poly A+ RNA was made, it is also theoretically possible that some of these clones arose from segments of genomic DNA which were ligated into the vector and mistakenly ended up in the cDNA library, although this is less likely.

In keeping with the absence of any mature heavy chain transcripts in the PK99H cells, the 8a5 clone itself appears to represent yet another aberrant VH sequence (figure 21). Interestingly, although the 5' end of clone 8a5 is identical to clone #2, and it encodes a protein essentially identical to the original PK99H heavy chain protein, it diverges from clone #2 by a three base deletion at codon +56 and a stop codon at +86, and the cloning linker appears beyond codon +87 (figures 15 and 21). Although this 8a5 clone was generated by PCR using a degenerate 5', V-region specific primer together with a Cγ1 specific C-region primer, the C-region primer does not appear in the

final cloned fragment, perhaps because of aberrant priming during the PCR, or perhaps because this portion of the PCR fragment was removed by the action of an exonuclease prior to ligation into the vector.

Problems identifying the 'true' P antibody VL cDNA- During our attempts to isolate this light-chain cDNA from cloned VL fragments generated by inverted RT-PCR, we obtained several sequences, including: 1) an aberrant light chain cDNA known to arise from the myeloma fusion partner (sequence I, figure 31), 2) an apparently normal, mature VL cDNA (the majority of the 15 clones analyzed; sequence III, figure 31), 3) apparent recombinants between sequences I and III (sequence II, figure 31), and 4) an aberrantly spliced cDNA arising from sequence III, which joins the -4 codon to the +55 codon, and splices out the intervening codons (sequence IV, figure 31).

NS-1 was the fusion partner for the PK99H, B, D, and E hybridomas, while SP2/0 was the fusion partner used in the creation of the P hybridoma. According to Carroll *et al.* (317), the gene responsible for the aberrant light chain transcript is present in both the NS-1 and SP2/0 myeloma cell lines. This is consistent with our finding that exactly the same aberrant light chain cDNA was cloned from both the PK99H and P hybridoma cell lines. Carroll *et al.* (317) also demonstrated that the level of transcription of the aberrant light chain varies between different hybridomas, with the level in some cases exceeding that of the 'expressed' light chain. Presumably PK99H could represent an extreme example of this, where there is no 'expressed' light chain transcripts remaining. The relatively higher abundance of the aberrant cDNA in the P hybridoma compared to B, D, and E may simply reflect the variation in relative levels of aberrant versus expressed light chain transcripts between these four different hybridomas. The fact that the P hybridoma is the only one of the four which arose from a fusion with SP2/0 probably does not explain the higher levels of the aberrant light chain cDNAs present in this hybridoma.

It is likely that the recombinant sequences which contain the 5' portion of the 'expressed' P antibody V_H joined to the 3' portion of the aberrant light chain (sequence II, figure 31) represent a PCR artifact, with the 'recombination' event arising during the PCR amplification. The simplest explanation for this finding is that incompletely copied segments of the expressed V_L sequence acted as primers on aberrant V_L sequences in the subsequent PCR cycle. Interestingly, the 'recombination event' occurs somewhere between codon +51 and +68 (figure 31), which represents the single longest stretch of identity (49 nucleotides) between the expressed V_L and aberrant V_L sequences. The relatively high ratio of aberrant V_L transcripts (4 of 15 clones) to expressed V_L transcripts (8 of 15 clones), together with the fact that the aberrant and expressed V_L DNA sequences happen to be highly similar, both would tend to favor such a 'PCR recombination' event. Presumably the reciprocal 'PCR recombination' event (e.g. the 5' portion of the aberrant light chain joined to the 3' portion of the 'expressed' P antibody V_H) would also occur, but this would perhaps be less favored since it was not detected among the clones analyzed. Shuldiner *et al.* (325) suggested that these 'PCR recombination' events would occur whenever PCR is used to amplify a gene that is a member in a family of genes with similar sequences , and a good example of this are the V-gene families. It may be possible to minimize these PCR artifacts by using longer extension times in the PCR cycle, to ensure that each strand is amplified to the very end. In our case the problem arises most often when the sequence of the expressed V_L is highly similar to that of the aberrant V_L. Again single-cell cloning and selection for a high-antibody producing hybridoma prior to RNA harvesting should ensure that there are relatively high levels of the expressed V_L transcript compared to the aberrant V_L transcript. In any case, the possibility of generating 'PCR recombination' events must be considered whenever PCR is used as the method to obtain V-region cDNAs.

One of the clones isolated during our attempts to clone the P antibody V_L cDNA likely represents an unusually spliced version of the expressed V_L (sequence IV, figure

31). In this case the usual signal sequence splice donor (normally splits the -4 codon; see open arrow-head on figure 31) splices to a splice acceptor (AG; circled with dots in figure 31) found at the junction of the +54/+55 codons. The presence of this particular splice acceptor sequence in precisely the necessary position to generate the final IV sequence by splicing makes it highly unlikely that the IV sequence arose from a PCR artifact. In any case, it would appear to be a minor transcript, since it occurred in only one of the 15 clones analyzed. Interestingly, the sequence appears to maintain the open reading frame, although it is unclear if a 'truncated' light chain would arise from this coding sequence.

Classical cDNA cloning and library screening versus inverted-RT-PCR to obtain V-region cDNAs- Clearly we were able to isolate V-region sequences using both methodologies, provided that the necessary V_H and V_L transcripts were present in the original mRNA sample. In comparison to the construction and screening of two cDNA libraries (which took several months, in part because of difficulties with the PK99H clones as described above), I-RT-PCR was very fast and simple, allowing the isolation of the D, E, and P sequences simultaneously over a period of weeks. The reason for our reluctance to use a PCR-based cloning technique in the initial experiments with the B and the PK99H antibodies arose from our desire to minimize the number of base-substitutions (e.g. mutations) due to *Taq* DNA polymerase, as well as to be free of the limitations on the cDNA sequence ends imposed by the use of degenerate 5' PCR primers, as explained in Chapter 1. However, once a high-fidelity thermostable DNA polymerase (*Pfu*) became available (which produced very few copying errors in comparison to *Taq* DNA polymerase) and once it became clear that by using inverted-RT- PCR we could locate both PCR primers outside of the V-region, and therefore impose no changes on the original V-region cDNA, we chose to adopt this approach. Our motivation for establishing the I-RT-PCR approach also related to our need to speed up the process of engineering antibodies. In fact, with the exception of the P light chain,

the I-RT-PCR approach allowed us to obtain and characterize the D, E, and P V-region cDNAs very rapidly. I-RT-PCR is clearly the method of choice for all future projects of this type.

Because we obtained three representative V_L and three V_H cDNA sequences for each antibody (sequences which in theory would be expected to be identical) and because we used both cDNA libraries (e.g. the B antibody sequences; where mutations would be due almost entirely to reverse transcriptase) and the I-RT-PCR method (e.g. the D, E, and P antibody sequences; where mutations would be due to a combination of reverse transcriptase errors combined with any errors generated subsequently during PCR amplification), we were in a position to compare the total number of copying errors which arose by either method. The fundamental idea here is that when three independent V region sequences are obtained, all are expected to be identical. Wherever the three sequences are not identical, the base which occurs in two of the three clones is assumed to be correct, and the 'non-consensus' base is assumed to be due to a copying error. The most common type of copying errors are base transitions (Elliott, personal communications): purine to purine (e.g. dG to dA), or pyrimidine to pyrimidine (e.g. dC to dT). One base-transition was detected in BV_H, where codon +103 was CGG (R) in one clone and TGG (W) in the other two clones, and no mismatches were found in the three BV_L clones. Since each V-region is roughly 300 bp in length, this means that the apparent error rate is 1 per 1800 bases copied (6 clones x 300 bases/clone were analyzed). For the clones generated by I-RT-PCR, in DV_H a T to C transition occurred in codon +112, with no disagreements between the three DV_L sequences; in EV_L an A to T transversion occurred in codon +84 of EV_L, with no disagreements between the three EV_H sequences; and in PV_H a T to C transition occurred at codon # 32, with no disagreements between the three PV_L sequences. This means that the apparent error rate is 3 per 5400 bases copied (3 antibodies x 6 clones/antibody x 300 bases/clone), or exactly the same as it was for the B antibodies, where no PCR amplification was

performed. Thus all of the 'mutations' which occur by either method appear to result from reverse transcriptase copying errors, and relatively few if any mutations are added during the PCR step. These results provide yet another argument for using the I-RT-PCR approach in all future projects of this type.

C. *Classification and analysis of V-region sequences with respect to somatic mutations and antigen binding affinity*

In an attempt to classify V_H and V_L genes, researchers began by grouping these proteins according to their amino acid sequences (see Kabat *et al.* (118)). Murine V_H-gene segments were divided into 3 subgroups based on protein sequences; later on these subgroups were further divided into 15 families based on nucleotide sequences (see (145) for review, (279)). V-sequences that share greater than or equal to 80% of their nucleotide sequence belong to the same family, whereas those that are less than 70% identical (ypically 20-60%) belong to separate families (279). The situation is similar, although not as straightforward, for murine V_K sequences (326), where 19 families have been assigned to 7 subgroups (128).

We first attempted to classify our V-region cDNA sequences according to the known V-region families and subgroups. This information, together with additional information about each V-region sequence, is summarized in table 8. All (V_H and V_L sequence-related classifications)mbut the J-gene classification was obtained by using three different computer programs which were available on Kabat's www page (327). The three programs used were: 1) 'Find your family', which gives information on the most probable V-region family that the antibody in question belongs to, along with the number of detected mismatches between the germline sequence for that V-region family and the query sequence; 2) 'Seqhunt II', which aligns the amino acid sequence in question with the most homologous antibody sequence present in the Kabat database, and also supplies information on the antigen specificity, isotype, and codon numbering scheme for the homologous antibody; and 3) 'Subgroup', which searches to match FRI

Table 8. Classification of V region families, CDR loops, unusual residues, Kabat sub-groups and J-genes for antibodies characterized in this thesis.

		V-Family	# of mis-matches between our Ab and the corresponding V-family (genomic sequence)	J-gene (mis-matches)	Unusual Residues-position, amino acid and % of all heavy chains with this residue in this position	Antigen specificity of the closest antibody sequence in the data base (isotype) [number of differences]	Kabat Sub-group	Chothia canonical classes of loop structure		
								loop # 1	2	3
B	V_H	VII	16	JH2 (2)	46=D-0.60% 62=M-0.24% 80=I-0.69% 101=V-0.64% 102=D-0.53%	Anti-DNA (IgG2b-k) [26]	IIB (1)	1	2	*
	V_L	XIII	14	Jκ1 (1)	8=I-0.02% 34=K-0.05% 93=I-0.57%	Anti-blood group A substance (IgM-k) [9]	V (1)	2	1	1
D	V_H	XIV	1	JH3 (0)		Anti-influenza virus(A/PR/8/35) hemagglutin (from primary response) [15]	IIID (0)	1	3	*
	V_L	XVII	0	Jκ2 (0)	80=Y-0.78% 83=M-1.00%	Anti-human transferrin receptor (IgG1-κ) [3]	V (0)	2	1	1
E	V_H	VIII	6	JH4 (0)	68=k-0.42% 72=V-0.40%	Anti-rheumatoid factor (IgG2b-κ) [12]	IIA (1)	1	2	*
	V_L	IV	0	Jκ1 (0)		Anti-fluorescein (IgG1-κ) [0] Autoantibody to thyroglobulin (IgM-κ) [0]	II (0)	4	1	1
P	V_H	VII	8	JH4 (0)	81=L-0.24%	Anti-DNA (IgM) [19] Anti-DNA (IgM-κ) [20]	IIA (0)	1	2	*
	V_L	IX	0	Jκ1 (0)	76=D-0.73%	Anti-DNA (IgM-κ) [1]	III (0)	?	1	1

* CDR3 of the HC can't be classified according to Chothia system ? Could not be classified because of length of loop Information in columns 1, 2, 5 and 6 were obtained using computer programs found in Kabat's www page (327): column 1 and 2 using 'find your family', column 5 using 'Seqhunt II' and column 6 "Subgroups' with (# of mismatches). The information in column 4 and 7 was obtained using programs in 'Antibody Sequence Test Results' (328) Colomn 3 (118).

of the antibody in question with Kabat's subgroups, highlighting the number of detected mismatches. Assignment of the J-genes was done manually, by comparing our J-sequences to the four known JH and five known JK genomic sequences (118). Finally, using information supplied through Kabat (327) and Martin (328) www page, the four light chain and four heavy chain V-region protein sequences were each aligned together, and the six CDR regions delineated so that the CDR loops could more easily be compared between the various antibodies, as shown in figure 57.

In terms of V-region families, it appears that our 4 different light chains fall in 4 different families, and only two of the heavy chains (B and P) can be placed in the same family. What is perhaps more interesting is to examine the number of mis-matches between our V-region sequences and the corresponding genomic V-family sequences. In general the D, E, and P antibodies show relatively few changes from the germ-line sequences, no changes at all (e.g. 0 mismatches) for any of the light chains, and 1, 6, or 8 mismatches for the heavy chains. This contrasts significantly with the B antibody, where there are 14 mismatches for the light chain, and 16 mismatches for the heavy chain. This suggests strongly that the B antibody has undergone significant somatic mutation (e.g. affinity maturation), whereas the other antibodies have undergone very little somatic mutation, and what has occurred has been confined to the heavy chain V-regions. This is consistent with the fact that the B antibody is a mature IgG (and therefore has had the opportunity to undergo significant affinity maturation before, during, and after isotype switching)mwhereas the D, E, and P antibodies are all relatively 'immature' IgMs. It is also consistent with the fact that the B antibody has the highest antigen binding affinity of all the antibodies. In a similar vein, the E antibody, which has the second highest antigen binding affinity, has a relatively larger number of mismatches in its heavy chain (e.g. 6), and although the lower affinity P antibody has even more mismatches (e.g. 8), it might be related to the fact that these mismatches are in part due to allelic variation and/or that the VH of the E and P antibodies represent new

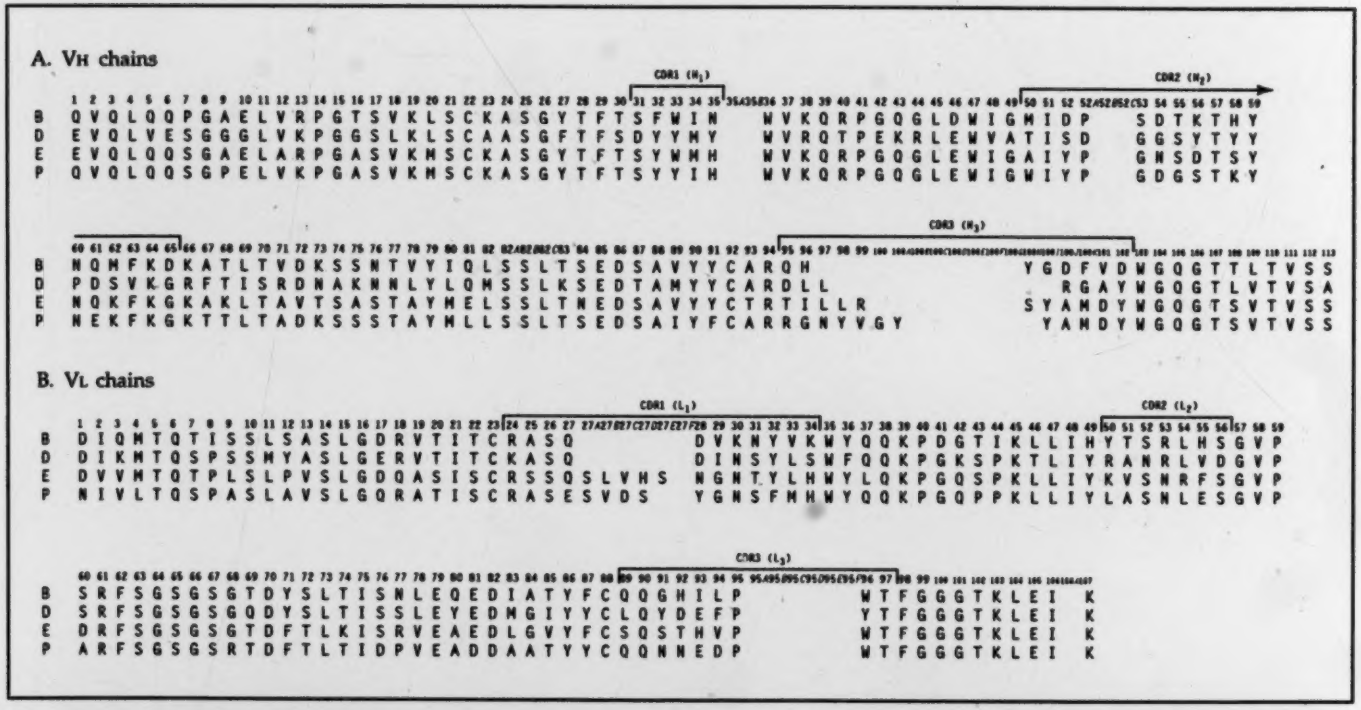


Figure 57. Amino acid alignment of VL and VH domains characterized in this thesis. Numbering is according to Kabat. CDRs were identified according to Kabat classification as follows: L1 (24-34),always begins after C, ends always prior to W. L2 (50-56) always starts at the16th residue after the end of L1, and the length is always 7 residues. L3 (89-97) always starts at the thirty-third residue after the end of L2, with the residue just before it being always a C, and the residues after always F-G-X-G. For heavy chain CDRs, H1 (31-35) always begins 9 residues after C22, and the residue after is always a W. H2 (50-65) always starts 15 residues after the end of H1. H3 (95-102) always starts at the thirty third residue after the end of H2 with a C-X-X always before it and WGXG always after it (3271,328).

germline genes which have not been previously cloned and sequenced, see below. The notion that the heavy and light chains of the B antibody have undergone significant affinity maturation is also seen by the fact that these V-regions have the largest number of unusual amino acids of the four antibodies studied, as shown in the fourth column of table 8. One question which arises as we attempt to classify our V-region sequences into the various V-families, and contemplate the significance of the number of mismatches vis-a-vi affinity maturation, is the following: How do we know that our V-region sequences do not represent examples of completely new members of V-gene families? Increased somatic mutation has been associated with isotype switching (IgM to IgG), however, limited somatic mutation is also known to occur in IgM antibodies (283). Because the entire human V-gene locus has been sequenced, all V genes are known, and this question does not arise for human antibodies. However, this is not yet the case for murine V-regions. In answering the question, two points must be considered: 1) the isotype of the expressed antibody from which the V-regions have been isolated (e.g. more changes due to somatic mutation would be expected in IgGs than in IgMs, both in the heavy and the light chain V-regions, which co-evolve in the same B cell), and 2) the locations of the mutations within the V-region sequence (e.g. mutations in J regions can be diagnosed with more certainty, since there are a limited number of mouse J region genes and they are all known). Strohal *et al.* considered this question for mouse light chain V-region sequences, and suggested that if 4-6 mismatches were found in a newly isolated V-gene from an IgM antibody, or if >30 mismatches were found in a newly isolated V-gene from an IgG, then the sequence likely represented a new member of the most homologous V-region family ((326), Kofler, personal communication). By this criteria none of our V_L sequences represent new family members. As for the V_H sequences of our IgM antibodies (D, E and P), mismatches present in E and P sequences might have been detected because the germline sequences present in these genes were not cloned before (e.g. V_H of E and P represent new germline genes). This is also

supported by the fact that no mismatches were detected in the accompanying light chains. In addition, the one mismatch in the D VH sequence might be due to somatic mutations, but a more plausible cause might be allelic variation. For the case of our IgG (the B antibody), we can assume that the VH and VL sequences of the B antibody underwent somatic mutations. These assumptions can be supported by the fact that mutations in the J region sequences apparently occurred only in the heavy and light chains of the B antibody while this was not seen in the other VH sequences.

D. *Analysis of V-region sequences with respect to antigen specificity and CDRs*

Table 8 also shows the antigen specificity of the antibody in the data base whose DNA sequence is closest to each of our V-region sequences, together with the isotype and the number of nucleotide mismatches between our V-region and the closest sequence. Two things can concluded from this analysis: 1) our V-regions are homologous to known antibodies having a wide range of antigen specificities, everything from DNA to carbohydrate blood-group antigens to viral surface glycoproteins. 1) In general our V-region sequences are not that close to any of the known V-region sequences (the light chains of E and P being an exception). This indicates that precisely the same monoclonal antibody has not been previously cloned and sequenced.

An alternate approach to analyzing our V-region sequences vis-a-vi antigen specificity is to focus exclusively on the CDR regions, which are known to form most or all of the antigen binding site. There are three CDRs present in the heavy chain, and three in the light chain (these have been designated simply H1, H2, H3, and L1, L2, L3 respectively). The protein sequences of the CDRs for our antibodies are shown in figure 57. Except for H3 and L1, all of the CDRs are the same length for all four antibodies. The B and D antibodies have relatively shorter L1 and H3 segments (11 amino acids for L1, and 7 or 8 for H3), whereas these same segments are longer in the E and P antibodies (15 or 16 amino acids for L1, and 11 or 12 for H3).

Based on structural information, Chothia *et al.* (124,329) determined that the H1, H2, L1, L2 and L3 loops can adopt a limited number of (canonical) structural conformations. In all, three different structural classes were recognized for H1, four for H2, five for L1 one for L2 and five for L3. (These investigators were unable to classify the H3 loops as falling into particular structural classes, due to the varying length and amino acid composition of this region; this is in keeping with the idea that H3 is highly variable, since it arises from the VH-DH-JH joining, and can have N-region additions, etc.). Moreover, Chothia *et al.* went on to show that the particular conformation adopted by any given CDR could be correlated simply with the length of the CDR loop and the presence of certain 'key' residues at specific positions within the loop. This allowed for the development of an algorithm by which the H1, H2, L1, L2 and L3 loops could be assigned to a particular structural class based solely on primary sequence information. In our case, by using the 'Antibody Sequence Test Result' computer program (328), we were able to assign the CDR loops of our various V-region sequences into their appropriate canonical structure classes, and this information is also summarized in table 8. The same program was also useful in identifying some of the 'unusual residues' located at various positions in the V-region sequences (table 8).

Vargas-Madrazo *et al.* (330) have systematically studied the various possible combinations of Chothia canonical structure classes for the H1, H2, L1, L2 and L3 loops , and they have attempted to correlate the presence of certain combinations of CDR structural classes with the general category of antigen recognized by the antibody. Theoretically there are 300 possible canonical structure class combinations; however, a sub-set of only ten of these combinations were found to encompass 86.9% of the 381 V-regions which Vargas-Madrazo and colleagues analyzed. Some of these 10 combinations are listed in table 9 (adapted from ref. (330)), along with the distribution of gross specificities for the known antibodies which fall into that structure class. Table 9 also shows the assignment of our antibodies to particular structure classes. Note that

Table 9. Distribution of some of the most frequent canonical structure classes and its correlation with gross specificities

		Canonical structure class (H1, H2, L1, L2, L3)	Percentage of antibodies falling in this class	Percentages of gross specificities for each class						
				Protein	Surface antigen	Poly-saccharide	Nucleic acid	Peptide	Hapten	specificity classification
P(?)		1-2-1-1-1	7.1	5	5	80	5	0	40	S
	B	1-2-2-1-1	24.5	16	44	0	18	0	23	M
		1-2-3-1-1	2.9	57	43	0	0	0	0	S
	E	1-2-4-1-1	14.2	11	4	5	24	52	4	S
	D	1-3-2-1-1	7.9	15	26	0	31	20	8	M

adapted from Vargas-Madrazo (330). 'S' stands for 'specific', and 'M' for 'multispecific'.

the structure class designation has been abbreviated (for example, to 1-2-2-1-1 for our B antibody, whose H1, H2, L1, L2, L3 loops fall into the structure classes 1, 2, 2, 1, 1 respectively table 8, last column). Note also that we were unable to assign the P antibody to a particular structure class (could be a member of any one of 5 classes) because assignment of the L1 loop was ambiguous. Essentially the refinement from 300 to 10 classes occurs because H1, L2, and L3 appear not to vary, and they always fall into canonical structure class 1 (this is not surprising in the case of L2, since there is only one structure class).

Based on whether or not the population of antibodies falling into any particular structure class demonstrated a predominant gross antigen specificity (e.g. >50% of the antibodies in that structure class recognized only one type of antigen), Vargas-Madrazo *et al.* (330) further classified the 10 predominant structure classes into two groups, 'specific' (=S) or 'multi-specific' (=M), as shown in the last column in table 9. It was found that in the 'specific' class there was a correlation between the length of the H2 and L1 loops and the type of antigen recognized. Short loops in H2 and L1 (the 1-1-2-1-1 combination) form flat binding sites that bind large antigens, whereas long loops in H2 and L1 (the 1-4-3-1-1 and 1-4-4-1-1 combinations) form a cleft, and tend to bind smaller antigens. Thus in the 'specific' class H2 and L1 apparently play the major role in determining the topography of the antigen binding site, and H3 assumes a more minor role. However this correlation does not seem to hold for the 'multi-specific' class (330), where H3 (which is not being analyzed) apparently plays the major role in determining the topography of the antigen binding site. To put this discussion in a broader context, as mentioned in chapter 1, it has been suggested that the topography of the binding site correlates with the type of antigen being recognized, with more flattened binding sites for larger antigens and more concave sites for the smaller antigens. Essentially this is in general what the Vargas-Madrazo *et al.* analysis does, only the approach has been refined, and hopefully made more precise, by assigning the CDR loops to particular

structure classes. For antibodies of the specific class, overall as one moves from the top to the bottom of table 9, the size of the antigen recognized decreases, from proteins to peptides to haptens.

Can any correlation with the type of antigen bound be obtained for our antibodies using the information in table 9. The B antibody falls in the 1:2:2:1:1 class, which is the largest class (accounting for 24.5% of the antibodies analyzed), these type of antibodies appear to bind primarily surface antigens (surface antigens>>haptens>nucleic acids≥proteins). This is at least consistent with the idea that the B hybridoma was raised against a surface antigen (pili), although because B falls in the multi-specific class, this type of analysis is clearly limited. It is interesting that two unusual residues (V and D; residues 101 and 102) occur at the end of H3, which is also consistent with a major role being played by this CDR in this antibody. The D antibody is classified 1:3:2:1:1 (7.9% of antibodies analyzed), which falls in the multi-specific group, and these type of antibodies bind predominantly to either nucleic acids or surface antigens (nucleic acids>surface antigens>peptides). The E antibody is classified 1:2:4:1:1 (14.2% of the antibodies analyzed), and interestingly it falls in the specific group, these type of antibodies recognize predominantly peptides, which is in keeping with the fact that the E hybridoma was generated against a synthetic peptide. As mentioned above, the P antibody could not be classified completely (it falls in one of 4 possible groups), since the L1 loop did not fall into any of the available Chothia canonical structure classes.

Although we have not taken this analysis any further, in theory it would be possible to attempt to predict which residues in our antibodies are the 'antigen contact residues', based on a detailed knowledge of the CDR loops and of the nature of the antigen which is recognized. In this context, by studying the crystal structures of antigen bound Fvs and Fabs MacCallum *et al.* (164) proposed a new definition for CDRs based on 'antigen contact residues', and developed methods to predict these based on the sequence of the CDRs the size of the antigen (e.g. a peptide is considered to be medium sized).

Determining the antigen contact residues would clearly be the next step in the process of humanizing our antibodies. The availability of computer programs to predicted antigen contact residues should allow 'best guesses' at how to humanize an antibody to be made rapidly, and some day these may replace the slower and more tedious 3-D computer modeling approaches.

To conclude this section, analysis of our V-region sequences using a variety of different computer programs provided us with information on the V-region genomic origin of these sequences and allowed us to make a correlation between affinity maturation, antibody isotype, and antigen binding constants. Information was also obtained on antigen binding sites (CDR length and amino acid sequence) which allowed us to place the antibodies in particular canonical structure classes. This information in turn was used to make some rudimentary correlation's with the type of antigen recognized. If at all possible, in any future work only antibodies of the IgG isotype, with proven high aKas (such as the B antibody) should be considered for chimerization or humanization.

E. *Eucaryotic expression vectors for the expression of mouse/human chimeric antibodies in COS cells*

In order to express mouse/human IgG3 chimeric antibodies, we constructed two 'γ3' version vectors, γ3.a and γ3.b. The γ3.a version vector contained introns on either side of the cloned V-region; that is, between our 'generic' (vector encoded) leader sequence (L) and the V-region exon (V), and between the V exon and the constant region exon (C). Preliminary results suggested that the V exon could potentially be simply 'skipped' in this vector, so that the L exon could splice directly to the C exon. In fact such an 'exon skipping' event would still lead to the production of a truncated C-region protein, because the splice-donor of the L exon (contributes first base of triplet) would generate an in-frame joining with the splice-acceptor of the C exon (contributes second and third bases of triplet), as seen in figure 36. Our experimental results were

consistent with this idea, since when transfected into COS cells, even the 'empty' γ3.a vector (e.g. prior to insertion of a V exon) generated supernatants which gave positive results by sandwich ELISA for human immunoglobulins. The same supernatants yielded a band on western blots which was shorter than expected for a full-length heavy chain. The problem was that this shorter band also appeared (though with less intensity and with an accompanying faint high molecular weight band) in supernatants from cells transfected with the γ3.a vector containing the B-antibody V exon (data not shown).

The idea of the failure of the L exon to splice to an inserted V exon in the γ3.a vector is somewhat surprising given that we have used precisely the same YPO4 exon + splice donor + intron + splice-acceptor element (figure 34) which has been used successfully before (Brain Seed, personal communication). In fact some portion of the transcripts might be appropriately spliced in the γ3.a vector once a V exon is inserted (the faint high molecular weight band mentioned above), but the problem becomes one of practicality, it is difficult to judge how much L to C splicing versus L-V-C splicing is occurring, and this might well vary for different V-region sequences, leading to different expression levels for different chimeric antibodies. At an even more basic level, if the empty vector gives a positive signal on sandwich ELISA, we cannot easily and rapidly test our cloned V constructs for expression.

We assumed that the problem with the γ3.a version vectors lay in the L to V splicing event. Therefore we removed this exon (e.g. by joining the coding segments of L and V directly into a single exon) to form the γ3.b version vector (figure 18). Fortunately, with the γ3.b version vectors, full length chimeric heavy chains could be generated whenever a V-region coding segment and downstream splice donor were inserted, whereas no protein was generated from the 'empty' vector. In fact the DNA segment between the V and C exons ends up being identical in both the γ3.a and γ3.b vectors, again emphasizing that this portion of the γ3.a vectors was fine, and the problem was in the L to V intron.

Interestingly, the same V exon 'skipping' event could potentially occur in naturally occurring antibody genes, in fact this splicing event has been detected in a naturally occurring, non-secretory human myeloma (331). In this paper the skipping was attributed to the 'modified structure' of the V exon. Another paper by Komori *et al.* (332) reported that V exon skipping can occur if the splice acceptor immediately 5' of the V exon was deleted. In addition Tanaka *et al.* reported on the discovery of 'exon recognition sequences' (ERS; usually purine rich) in M2 exon (encoding part of the transmembrane piece) in the μ constant region (333) (these sequences have also been found in other non-immunoglobulin genes) and were identified as splicing enhancers (333). How does this information about naturally occurring L-V-C splicing relate to the failure of our γ3.a vector to efficiently undergo L-V-C splicing? Three points need to be considered 1) our YPO4 L exon is not that of an immunoglobulin, it is the leader sequence of a human lymphocyte glycoprotein, 2) in the 'wild-type' immunoglobulin genes the V-exons are always found with their corresponding L-exons (they come as one unit, the V-exons containing the last 4 codons of the leader sequence), and 3) in the 'wild-type' immunoglobulin DNA the V-exons are always located between the leader and the constant region. Building on the above mentioned facts, we could hypothesize that the V-exons have to be present between the leader and the constant region exons in order for the 'correct' splicing to occur. This role of the V-exon in assuring 'correct' splicing could be achieved as a result of: 1) the closer location of the V to the leader exon in comparison with that of the constant region exons, and 2) the presence of certain ERS or ERS-like sequences in the L/V-exons that can work in combination (with some L/V combination working better than others) to assure L/V splicing and not L/C. This latter idea can be related to the observation of Gillies (334) that by exchanging an L exon, a certain light chain construct switched from a producer to a non-producer phenotype. Thus we might have been successful if we had inserted a genomic

fragment containing the naturally occurring unit of (L exon-intron-expressed V exon) of the B hybridoma, but this genomic cloning approach would be highly impractical.

Overall, our experiences with the γ3.a vector suggest that although inserting 'whole exons' (containing intron sequences) may be attractive in that the restriction sites can be 'hidden' in the surrounding introns and therefore be absent from the final spliced mRNA, introduction of these same restriction sites and other changes into the introns may affect splicing in a subtle and unpredictable fashion. This fact will also depend for a large part on the sequences of the exons themselves, since it was reported by Gillies *et al.* (334) that for some light chains the L-V intron was actually needed for expression. In any case, creation of the γ3.b series of vectors, where the coding changes due to the *Bam*HI site are imposed on the leader sequence rather than on the V-region (figure 40), appears to have been a highly successful solution which allowed us to proceed with the production of chimeric human IgG3 antibodies. Availability of the γ1.c version vector allowed us to also proceed with production of chimeric IgG1 antibodies, in this case, relying on the fact that the vector had been carefully designed to enable direct joining of the V and C coding elements, without inducing any coding changes.

Our rationale for making both IgG3 and IgG1 chimerics essentially reflected our desire to 'cover all possibilities' for passive immunotherapy of *Pseudomonas* infections. Both IgG3 and IgG1 are efficient at antibody mediated cell-lysis, and which of the two is most superior depends on the experimental conditions, such as antigen concentration, antigen 'patchiness', and complement concentration (216,217). In fact IgG3 appears to dominate under most conditions, except at high antigen concentrations (216,217). On the other hand, IgG1 is considerably more stable than IgG3. Finally, it is difficult to extrapolate from the *in vitro* results to the *in vivo* situation.

F. Analysis of the secretion of the IgG3 and IgG1 chimeric antibodies

After the isolation and sequencing of the V_H and V_L regions, V-region fragments were cloned into the different 'b' and 'c' version expression vectors. COS cells were

transfected with the V-containing expression plasmids, and supernatants collected and concentrated. The supernatants were tested in western blotting and competitive ELISA experiments. The westerns were performed to evaluate secretion and proper assembly, and the competitive ELISA data was used, as described by Nieto (322), to determine the apparent association constants (aKas) of both the chimeric and mouse antibodies.

Western blotting analysis of transfected COS supernatants under reducing conditions confirmed that the chimeric IgG3 and IgG1 antibodies were secreted, and that the corresponding heavy and light chains were of the expected sizes (figures 49 and 50). Similar analysis of the parental mouse monoclonal antibodies (figure 52) confirmed that the H and L chains were also as expected. The two light chain bands seen in the mouse P antibody (figure 50) likely arise because of differential glycosylation, a phenomenon which has also been observed for other monoclonal antibodies (302,335). Western blots of these same supernatants run under non-reducing conditions showed that the various immunoglobulins were being assembled, as evidenced by the disappearance of the monomer bands seen in the reducing gels, and the appearance of >97 kD bands. The molecular weight of assembled human IgGs are expected to range from 146-170 kD (193) (51-56kD contributed by the heavy chains and 25 kD by the light chains (119)), and although the exact molecular weight of the non-reduced bands was not determined, they are certainly within the expected range.

As presented in chapter 1, the heavy chain binding protein (Bip) found in the ER of COS cells (336) binds nascent heavy chains and retains them in the ER. The heavy chains are only released from the ER when the Bip is displaced by light chains during immunoglobulin assembly (337). In contrast, light chains can be secreted directly, without any requirement for heavy chains. Since the secretion of heavy chains is dependent on the binding of light chains, in our Western blots we would expect to see either: 1) heavy and light chain bands with relatively the same intensities, or 2) light chain bands being more intense than the heavy chain bands. In the latter case, we would

also expect to see some smaller bands on the corresponding non-reducing gels, which would represent the excess light chains, likely secreted as dimers or a mixture of monomers and dimers. These ideas appear to fit most, if not all, of our Western blot data. For example the IgG3 chimeric B antibody appears to have balanced heavy and light chain expression (figure 49) and shows no lower molecular weight bands on non-reducing gels (figure 49), whereas the IgG1 chimeric B antibody appears to have a clear excess of light chains over heavy chains (figure 49), and shows a strong lower molecular weight band on non-reducing gels (figure 49).

Besides balancing levels of heavy and light chain transcripts (e.g. by transfecting equimolar amounts of heavy and light chain expression plasmids into each COS cell), it may be that certain light chains are secreted more efficiently than others, and both of these phenomenon may be affecting the observed balance or imbalance of heavy to light chains in our transfected COS cell supernatants. As an extreme example, Horwitz *et al.* (338) compared the secretion of 12 chimeric light chains, and found that some of these were not secreted at all, whereas others were efficiently secreted, and these differences could be attributed to amino acid sequences in frame work region 1. However, since all of our light chains appeared to be secreted efficiently by themselves (see below), and since the IgG3 and IgG1 chimerics shown in figure 49, 50 both used identical light chains, the problem of balancing heavy and light chain expression appears to reside more at the transcript level. Alternately the differences in heavy/light chain balances between the IgG3 and IgG1 B antibody chimerics may reflect the differential capacity of the two different heavy chain isotypes to pair with that particular chimeric B light chain.

G. *Analysis of the binding affinities of the IgG3 and IgG1 chimeric antibodies*

In terms of classification of functional binding affinities, I came up with the high (H), moderate (M) and low (L) categories presented in tables 6 and 7 by considering the work of Doig *et al.* (105), who found that the mouse monoclonal antibody PK99H, which had an aKa of 300 ml/g, could effectively block *Pseudomonas aeruginosa* strain

PAK from binding to BECs (105), as well as passively protect mice from lethal injections with *Pseudomonas* (304). Thus the PK99H antibody was of sufficient affinity to be highly effective, and in comparing our chimeric antibodies to PK99H, we classified them as high (aKa ≥ 300 ml/g; e.g. aKa ≥ that of PK99H), moderate (aKa 100-300 ml/g, down to 1/3 of PK99H) and low (aKa <100 ml/g). These considerations are of course based on affinity alone, and do not take into account the fact that antibodies which bind certain epitopes may be practically useful in vivo, whereas those which bind other epitopes with equally high affinities may nevertheless have no useful effect.

Surprisingly, all of the chimeric IgG3 antibodies exhibited aKa values higher than those of the corresponding mouse antibodies (table 6). In part this may reflect the relatively poor quality of the binding data for the mouse (and some of the chimeric) antibodies (note the standard deviations on the I_{50} values). However, two main points can still be made from a consideration of the aKa values shown in table 6: 1) the B antibody maintains a relatively high aKa value in all cases, which is in keeping with the IgG isotype and affinity maturation seen in the original mouse antibody, and 2) the original mouse E antibody (which was the most protective in animal studies; R. Irvin, personal communication) shows a relatively high affinity, and this is maintained in the IgG3 chimeric, but disappears in the IgG1 chimeric. The discrepancy between the aKa values for the two different E antibody chimerics cannot be attributed to any V-region sequence differences between the γ1 and γ3 constructs, since the two expression plasmids were sequenced to ensure that no base changes had occurred during the PCR and subcloning process. In fact, in all of the subcloning only one base change was detected from the original V-sequences, [residue #48 in the D(V_H), with no change in the coded amino acid] giving an overall error rate of 1 in 20,000 for the *Pfu* amplification and subcloning process.

In a similar fashion to us, Man Sung Co and colleagues also constructed mouse/human chimeric IgG1 and IgG3 antibodies, using the same mouse V region in

each chimeric. These investigators obtained a somewhat different result than I did, however, since they found the affinities of the IgG1 chimerics to be consistently higher than those of the IgG3 chimerics (273). In our case, except for the E antibody it would be difficult to be certain that our IgG3 chimerics have a higher affinity than the IgG1 chimerics, although this certainly appears to be the trend for all antibodies tested. In terms of systematic differences between IgG3 and IgG1, Cooper *et al.* did report that IgG3 was a better binder than IgG1 at high but not at low epitope densities, and they attributed this to the fact that IgG3 had a faster association rate and a slower dissociation rate in comparison to IgG1 (339). This may be related to the greater flexibility of the hinge region in IgG3. In our case we could have investigated this question by doing competitive ELISAs on different forms of the antigen, such as PAK pili peptides conjugated to BSA as well as whole PAK pili, and by varying antigen density. Another possible reason for the differences between the binding affinities of the B antibody IgG3 and IgG1 chimerics may be related to the excess of light chains present in the IgG1 supernatants. Light chain dimers can also bind to antigen but with a lower affinity, and these could potentially make a contribution to the measured binding affinity of the 'IgG1 chimeric' antibody.

With the exception of the B antibody, in general the γ3 and γ1 vectors were comparable in terms of antibody secretion; thus we conclude that the presence or absence of an intron between the V and C exons did not seem to have a major effect on expression levels. For future projects, choice between the two expression vectors would depend primarily on which antibody isotype was desired. However, given our results with the E antibody chimerics (which we believe to be real), it would make sense to attempt to express chimerics of both isotypes, or at the very least, attempt IgG3 even if an initial IgG1 chimeric failed to bind antigen.

H. *Analysis of the secretion and binding affinities of singly expressed heavy or light chains*

COS cells transfected with the light chain constructs alone resulted in secreted light chains for all four antibodies (figures 49, 50, 53, and 54) , with B(V_L) and D(V_L) exhibiting moderate binding (table 7; note that the D(V_L)k.c protein would have modified amino acids at the V-C junction, as shown in figure 49, and this could affect L3 and antigen binding). Interestingly enough , both of these light chains belong to the same Kabat subgroup (V; table 8) and the same Chothia canonical structure class (2-1-1; considering only the L1, L2, and L3 loops shown in table 8). In comparison, singly transfected heavy chains resulted in secretion of only the D (figure 53, lane 8) and P (figure 54, lane 4) γ3 heavy chains, with high binding exhibited by the D(V_H)γ3 protein and moderate binding by the P(V_H)γ3 protein (table 7). None of the γ1 heavy chains were secreted in isolation (data not shown). These results suggest that certain unique combinations of V regions + γ3 constant regions can overcome the need for light-chain binding prior to secretion. There have been other reports of transfected cells secreting heavy chains without an accompanying light chain, although the γ3 isotype was not commonly used in these experiments. Some of these reports attribute the capacity for isolated heavy chain secretion to deletions in the C_H1 domain (e.g. removal of the Bip binding site; ref. (180)), while others attributed it to the presence of extremely low, essentially undetectable, levels of light chain protein (340). The latter theory cannot be reconciled with at least one report of isolated heavy chain secretion from a cell line where transcription of the light chain genes was completely blocked at the mRNA level (334).

I. *Analysis of the secretion and binding affinities of chimeric antibodies resulting from light chains exchange or V-domain swapping*

These two approaches were used in order to improve and evaluate the binding affinity of D and P chimeric constructs. In the case of the P antibody, we expressed the γ1 chimeric protein with all of the other available chimeric light chains. Motivation for this 'light chain exchange' experiment was three fold: 1) the fact that P antibody γ3 chimeric heavy chain could be secreted in isolation but exhibited only moderate binding,

2) the fact that we had encountered some difficulty identifying the VL cDNA for the P antibody (thus we at least entertained the possibility that we had not cloned the 'correct' VL) although we had cloned the predominant VL mRNA, and 3) its' sheer simplicity. In the case of the D antibody, we were impressed that the D-γ3 chimeric chain could be secreted without a light chain, and moreover that this single heavy chain demonstrated high binding affinity. Therefore we chose to do a 'V-region swapping' experiment on the D antibody. Besides testing new approaches and potentially yielding interesting results, we anticipated that these experiments might improve our understanding about which CDRs in these antibodies were the major contributors to antigen binding.

The attempt at improving the aKa of the chimeric P antibody was very successful. The basic idea was to 'rescue' the heavy chain by providing a more 'well-behaved' light chain. Western blots showed that all antibodies were secreted and assembled (figure 54). Best results (e.g. highest aKa values) were obtained when the B and then D light chains were used. Interestingly, these two light chains showed moderate binding when expressed in isolation. The binding affinities clearly do change when the various different light chains are combined with the P heavy chain (table 7), and looking at the Chothia canonical structure classes of the newly formed antibodies, it is clear that the 1-2-2-1-1 combination was formed with both the B and D light chains, placing these particular heavy and light chain combinations in the same structure class as the B antibody. Similarly, the combination with the E light chain produced the same structure class (1-2-4-1-1) as the E antibody. Similar results have been reported recently by Ichiyoshi and Casali (341) arising from their studies of the determinant factors in IgM polyreactivity. In 1996, Ohlin *et al.* also used this same light chain shuffling approach combined with phage display technology to modify the affinity of a particular antibody (342). In conclusion, light chain shuffling provides a rapid and convenient approach to potentially rescue 'failed' chimeric antibodies, particularly if you have a number of light chains constructs sitting in your freezer. If time permitted we could have done the same

type of experiment with the B, E and D heavy chains as well, if possible also making use of information about the fine specificity of these antibodies. Given that the B mouse antibody is 'non-protective', it might be especially interesting to see if by combining the light chain (wild-type or chimeric) from one of the other 'protective' antibodies with the (wild-type or chimeric) B heavy chain a 'protective antibody' could be generated.

The V-region 'swapping' experiments were successful in expressing the desired constructs (figure 53). However, they did not provide clear information on the contribution of the different H and L CDRs to antigen binding, and they yeilded proteins with affinities comparable to that of the mouse D (and IgG1 chimeric) antibody but not significantly higher. Perhaps if the same approach had been applied to our B antibody it might have been more successful, since B has a higher aKa and it is multispecific, the same as D, which means that H3 is a major contributor to the topography of the antigen binding site. One of the potential problems with our cloning approach to V region swapping is that we are changing the first few amino acids in the C regions (figures 55 and 56) which could potentially have an effect on the structure of the molecule and/or on glycosylation patterns, which would in turn affect antigen binding (191,343). V-region swapping has been done successfully by Sharon et al (344), who used genomic DNA, whereby V_H was spliced to C_k, which probably resulted in no amino acid changes in the constant region. Sharon's V_H-Ck molecule associated with the wild-type V_L-Ck light chain from the same antibody, thus forming a 'heterodimer' which retained high affinity binding of the antigen.

Several conclusions and future directions can come out of this work:

1) New chimeric antibodies with high affinities for *Pseudomonas aeruginosa* pili have been produced. These antibodies are some of the first to be produced in the *Pseudomonas aeruginosa* field.

2) New antibody sequences have been isolated and characterized for the first time; some may even represent new germline genes that have not been cloned before.

3) Some of the isolated V-regions are currently set for further 'humanization' to be used in human clinical trials as prophylactic therapeutics, as well antibody reductants (antibody fragments that retain antigen binding capabilities) are to be made (Irvin, personal communication).

4) The light chain exchange approach was very successful in rescuing desired heavy chains and in modulating antibody affinity. This technique could be further applied to provide a closer look at antigen specificity and to further improve the D and maybe E binding affinity.

5) Inverted RT-PCR is the method of choice for the isolation of V-region cDNA. This attempt represents one of the first to use the inverted PCR to isolate immunoglobulin sequences. With the use of the 'tailor-made' primers hybridizing to the 5' and 3' ends of the constant region sequence; no base changes were detected as a result of the amplification process.

6) New expression vectors were created containing all the necessary signals and regulatory elements required for the expression of chimeric IgG3 antibodies. V-regions can be easily cassetted with no imposed changes on either of the 5' or 3' ends.

7) Placement of the restrictions sites used to insert V-region sequences within the V/C intron is very useful, and has no deleterious effects on protein expression. However, if an L/V intron is to be used it may be necessary to isolate the entire genomic unit of L-intron-V to eliminate direct splicing of a synthetic L exon to the C exon.

8) If the affinity of the resulting IgG (γ3 or γ1) chimeric is lower than that of the parent mouse antibody, it may still be very useful to try and re-engineer that same V-region onto the other human gamma constant region, keeping in mind that the in vitro assays for affinity that one might be using may not reflect in vivo conditions.

9) This work also confirms the possibility of solitary heavy chain secretion (as was the case for some of the γ3 chimerics), and the possibility that dimerized heavy chains alone

may retain significant antigen binding capability. This also confirms the unequal contributions made by the H and L CDRs to antigen binding.

10) V-region swapping could also be applied to for testing the H and L chain CDR contribution to antigen binding for the E, B, and P antibodies. However, applying this to the B antibody first might give a better evaluation of the technique since B has a very high affinity and as with the D antibody, the B is of the 'multi-specific' class which means that the CDRH3 is a major contributor to antigen binding.

11) It is more practical to work with IgG antibodies that exhibit high affinity rather than high avidity IgM antibodies.

12) Emphasis should also be placed on the need to re-clone the hybridoma cell line continuously for selection of high producer cells, especially prior to RNA isolation.

13) Methods described in this thesis could also be used to isolate V-region sequences from 'important' hybridomas not just for the sake of engineering, but rather to permanently obtain the V-region sequences before the hybridoma loses the immunoglobulin genes due to hybridoma instabilities.

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APPENDIX

Competitive ELISA profiles of the mouse antibodies and the chimeric constructs

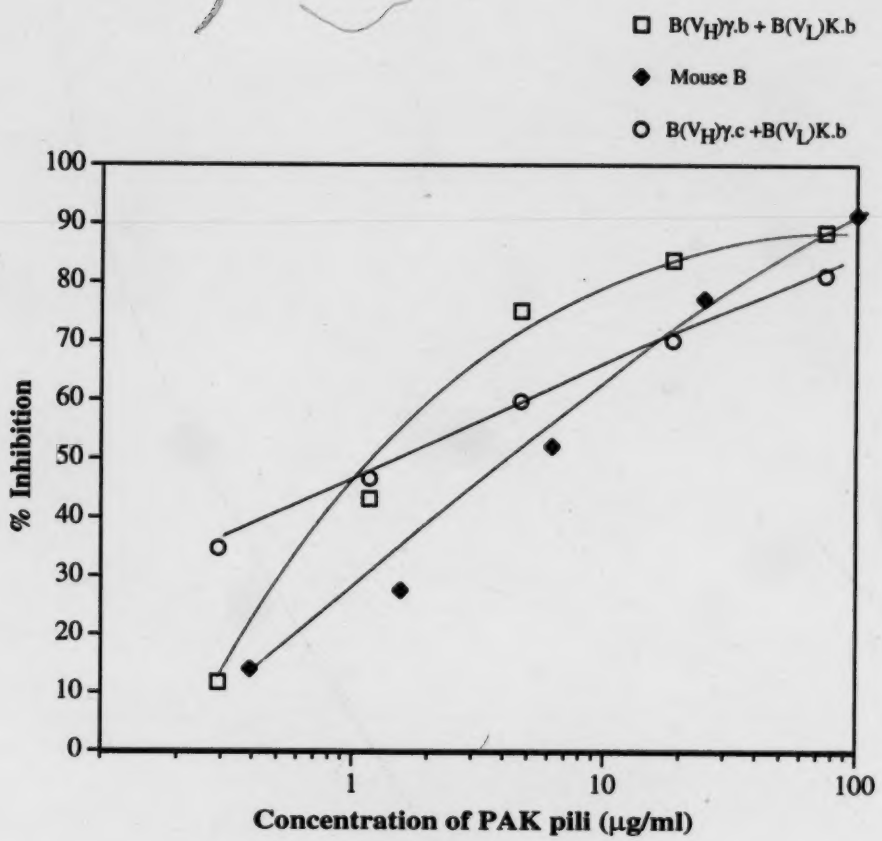

Figure A-1 Representative competitive ELISA profiles of B antibodies showing the inhibitory effect of adding non biotinilated PAK pili on the binding of B to biotinilated PAK pili.

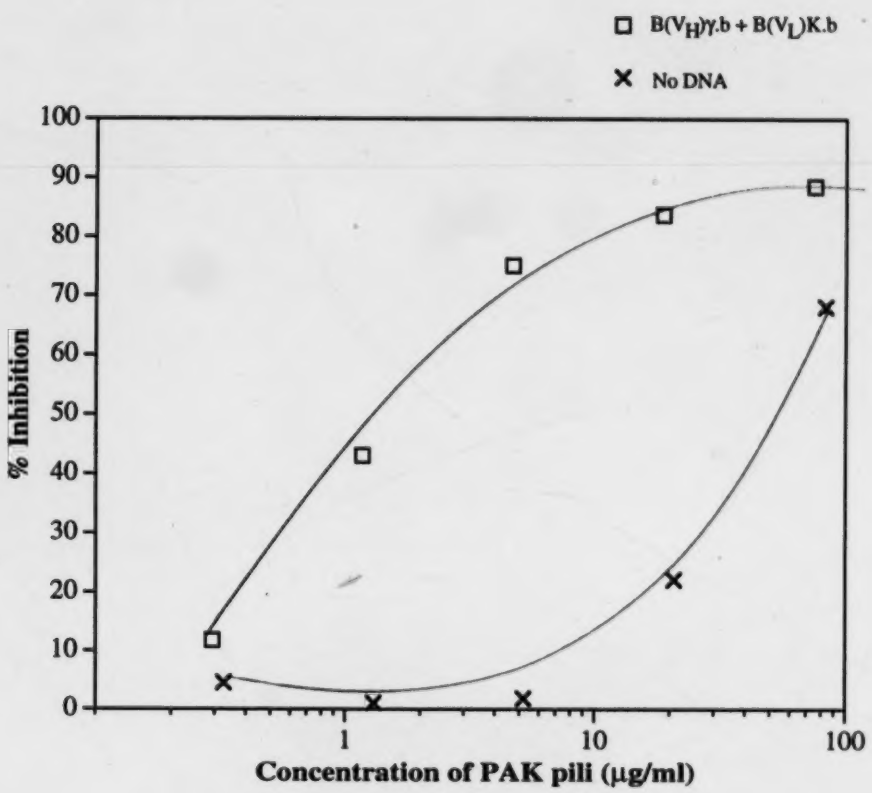

Figure A-2 .A competitive ELISA profile of B chimeric gamma 3 antibody in comparison with 'soup' from a transfection experiment where no DNA was used.

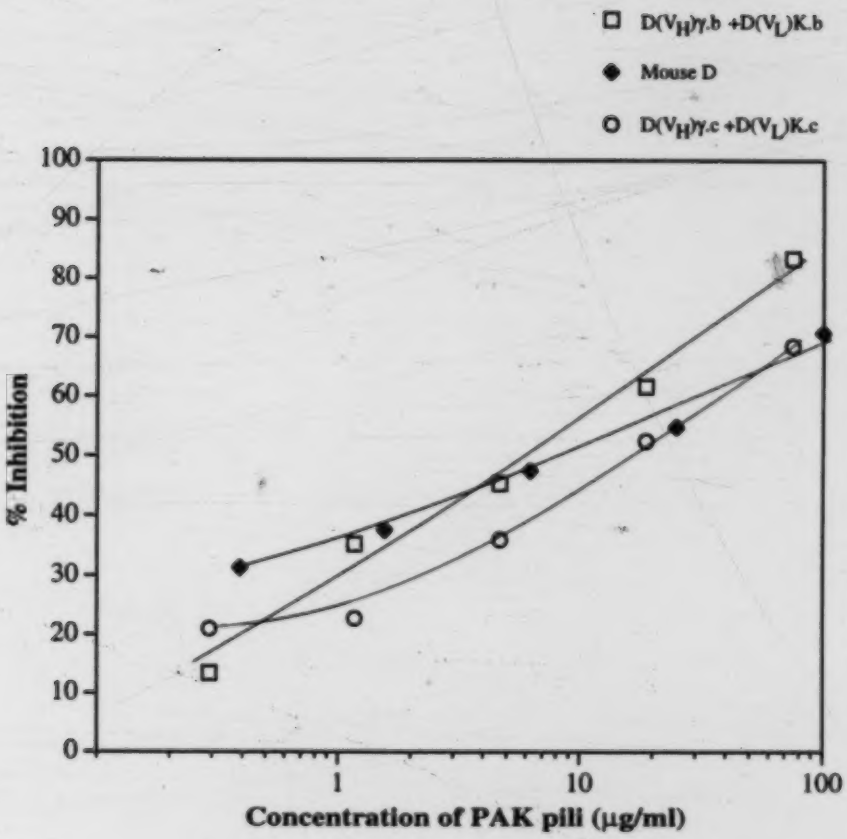

Figure A-3. Representative competitive ELISA profiles of D constructs showing the inhibitory effect of adding non biotinilated PAK pili on the binding of these constructs to biotinilated PAK pili.

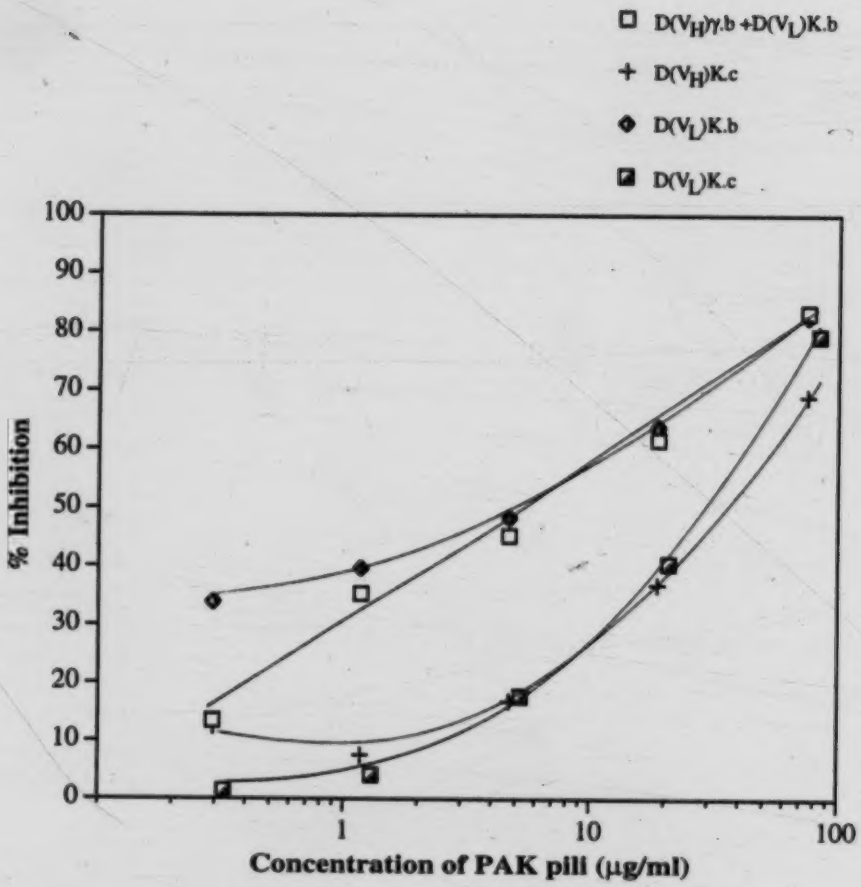

Figure A-4 Representative competitive ELISA profiles of D chimeric constructs. The figure shows the inhibitory effect of adding non biotinilated PAK pili on the binding of the constructs to biotinilated PAK pili.

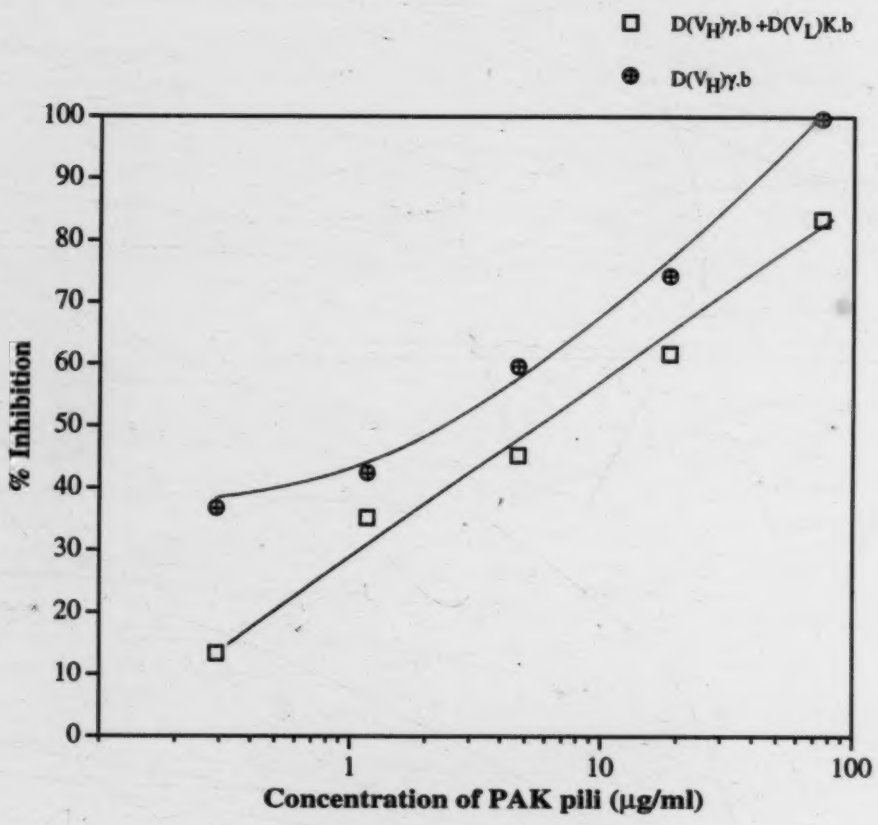

Figure A-5 Representative competitive ELISA profiles of D gamma 3 chimeric antibody and that of the chimeric gamma 3 heavy chain by itself The figure sh ow the inhibitory effect of adding non biotinilated PAK pili on the binding of the constructs to biotinilated PAK pili.

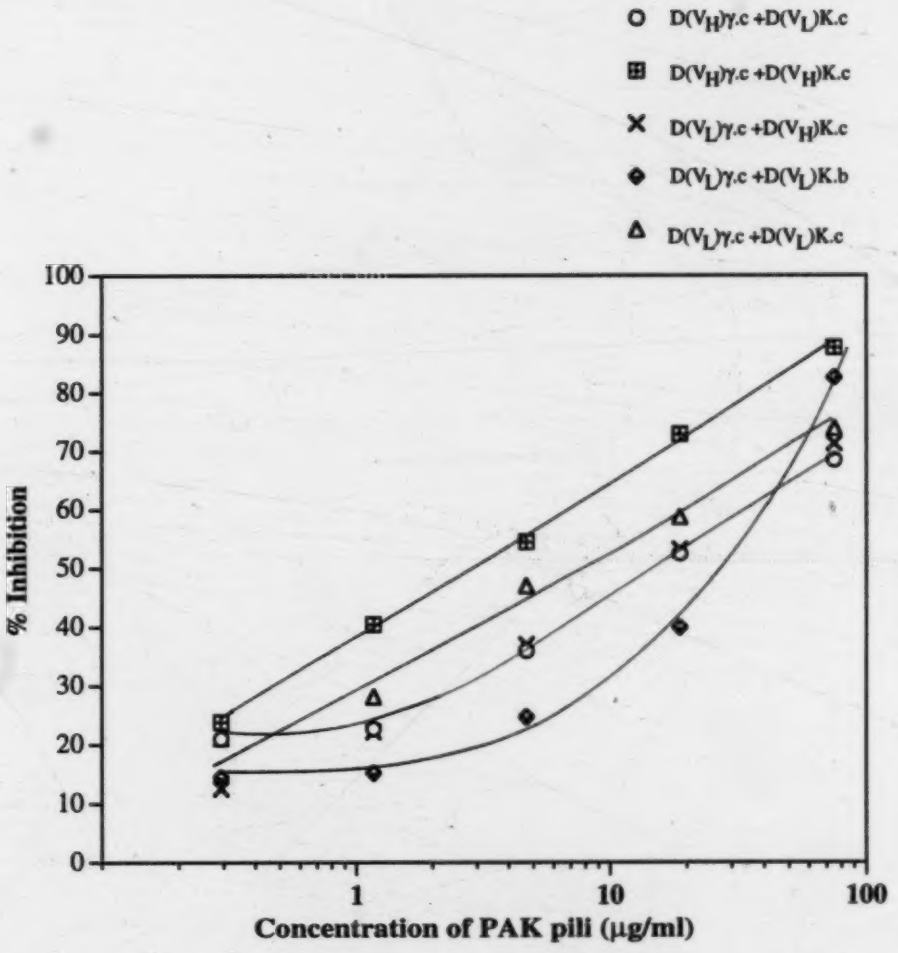

Figure A-6 Representative competitive ELISA profiles of D chimeric gamma1 with variable region-switch constructs. The figure show the inhibitory effect of adding non biotinilated PAK pili on the binding of the constructs to biotinilated PAK pili.

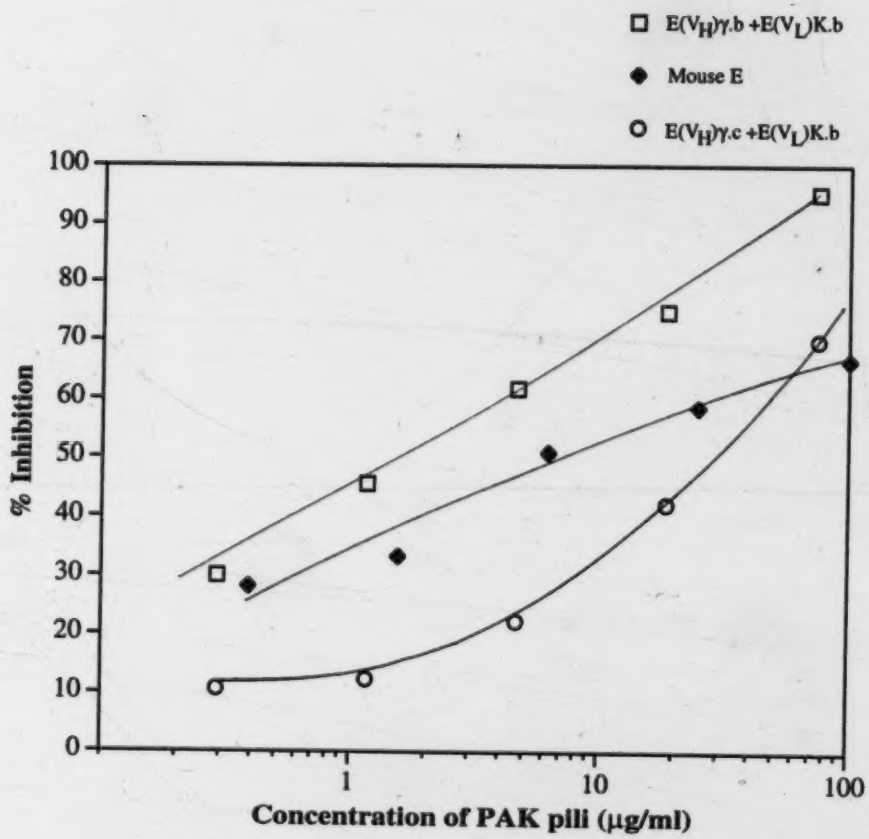

Figure A-7 Representative competitive ELISA profiles of E constructs showing the inhibitory effect of adding non biotinilated PAK pili on the binding of E constructs to biotinilated PAK pili.

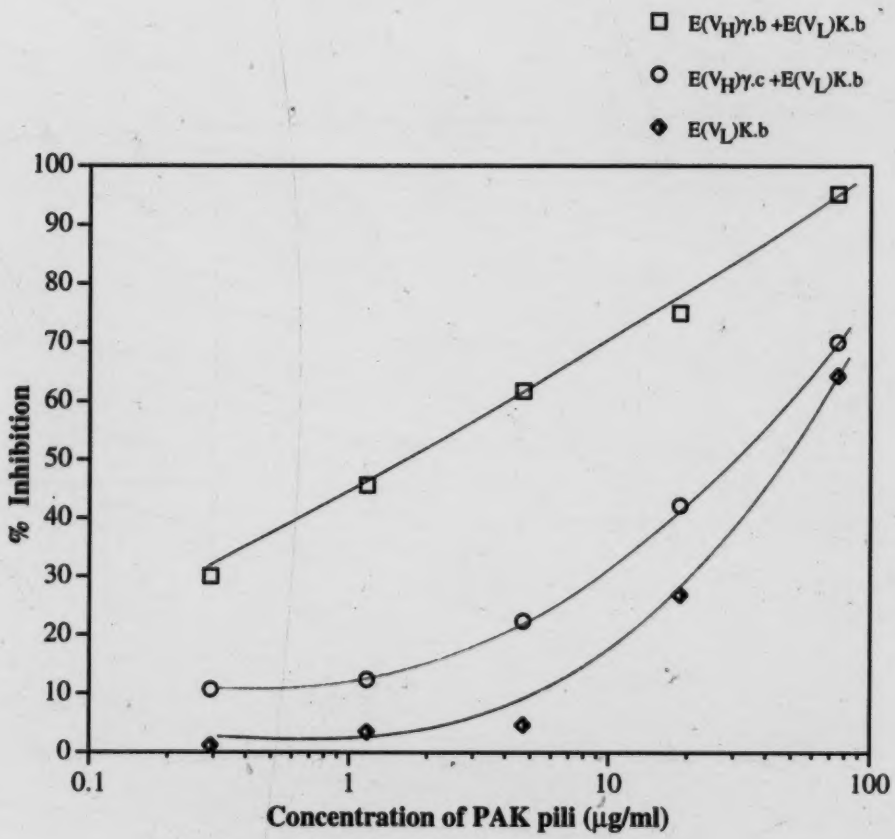

Figure A-8 Representative competitive ELISA profiles of E chimeric constructs showing the inhibitory effect of adding non biotinilated PAK pili on the binding of E constructs to biotinilated PAK pili.

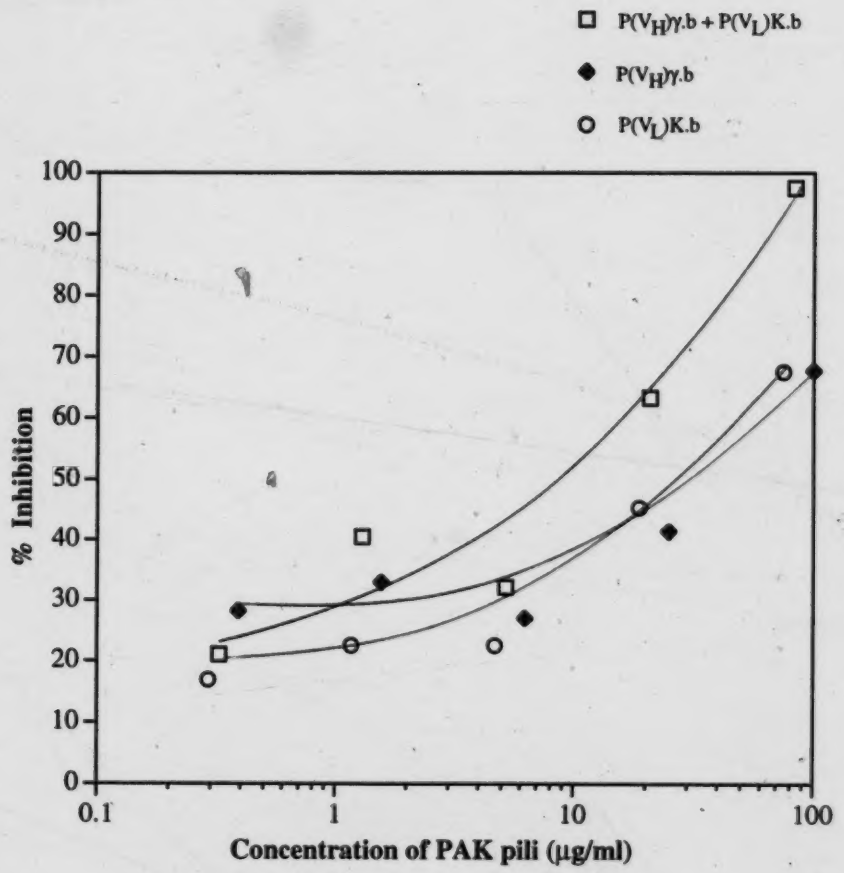

Figure A-9 . Representative competitive ELISA profiles of P antibody constructs, showing the inhibitory effect of adding non biotinilated PAK pili on the binding of P constructs to the biotinilated PAK pili.

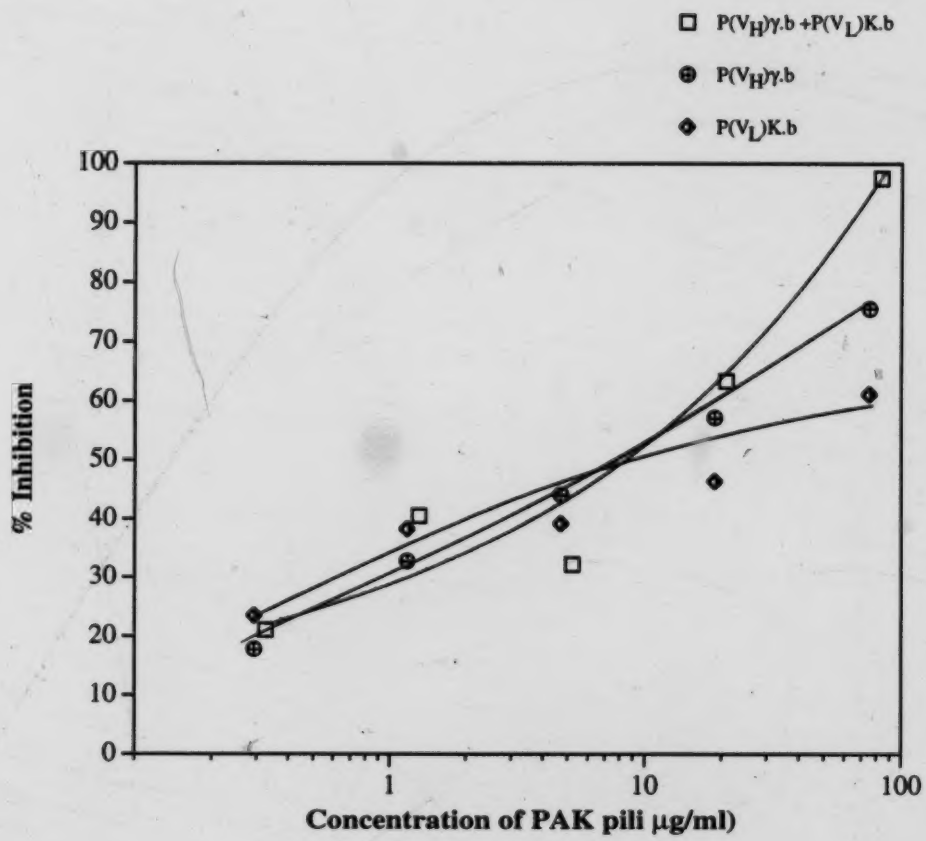

Figure A-10 Representative competitive ELISA profiles of P antibody constructs showing the inhibitory effect of adding non biotinilated PAK pili on the binding of these constructs to biotinilated PAK pili.

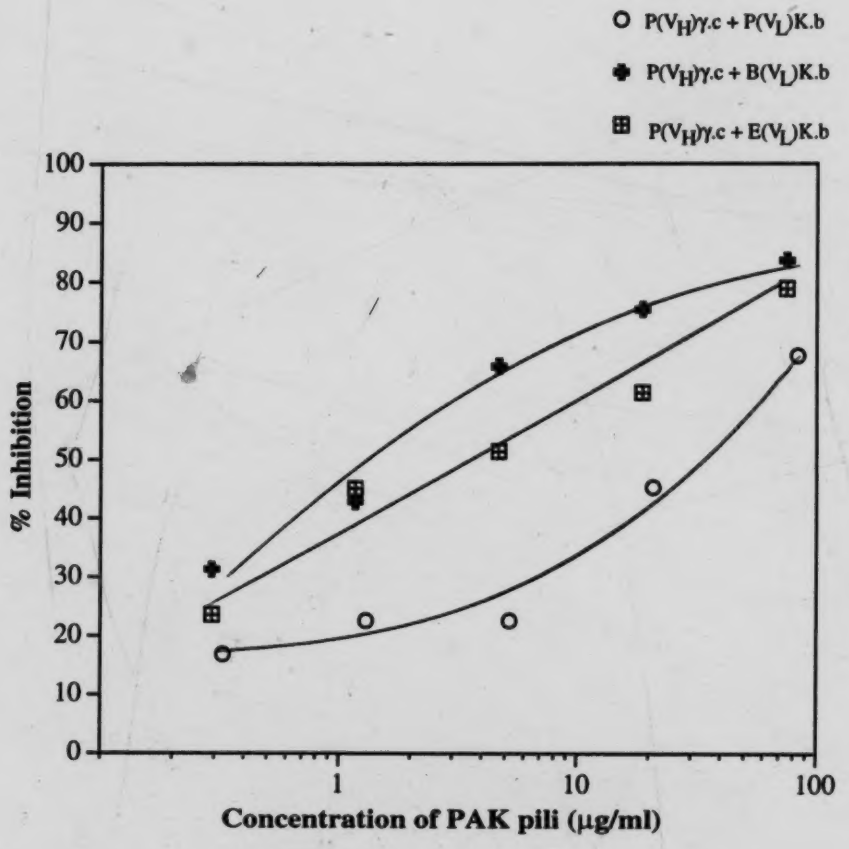

Figure A-11 Representative competitive ELISA profiles of P chimeric gamma 1 heavy chain in combination with its own and other light chain. The figure show the inhibitory effect of adding non biotinilated PAK pili on the binding of these antibodies to ibiotinilated PAK pili.

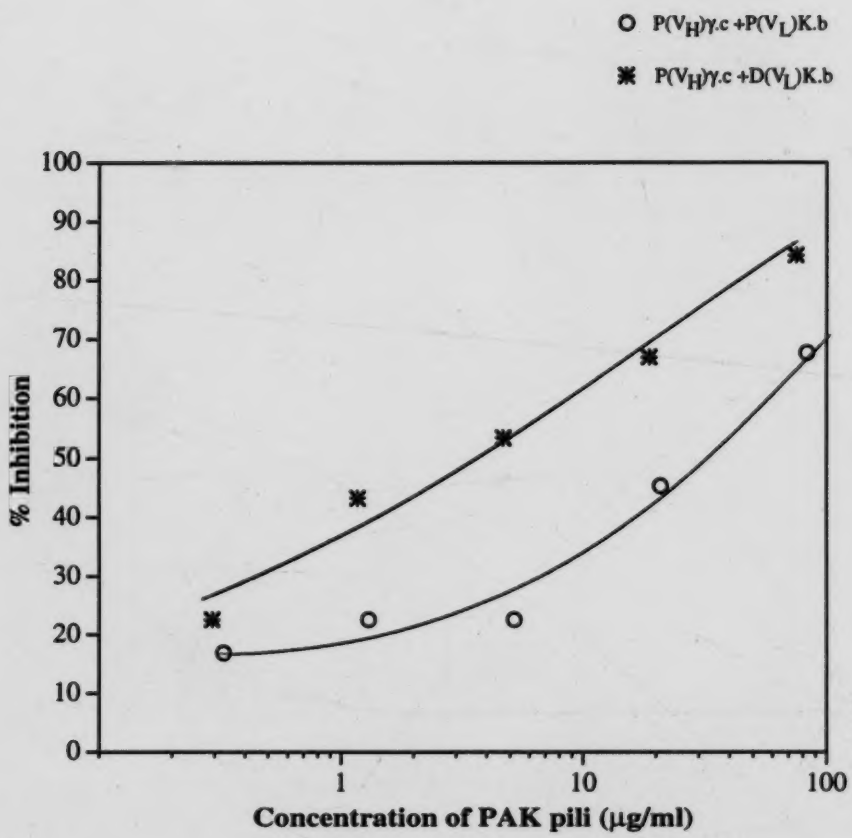

Figure A-12. Representative competitive ELISA profiles of P chimeric gamma 1 heavy chain in combination with its own and D light chain. The figure show the inhibitory effect of adding non biotinilated PAK pili on the binding of these antibodies to biotinilated PAK pili.